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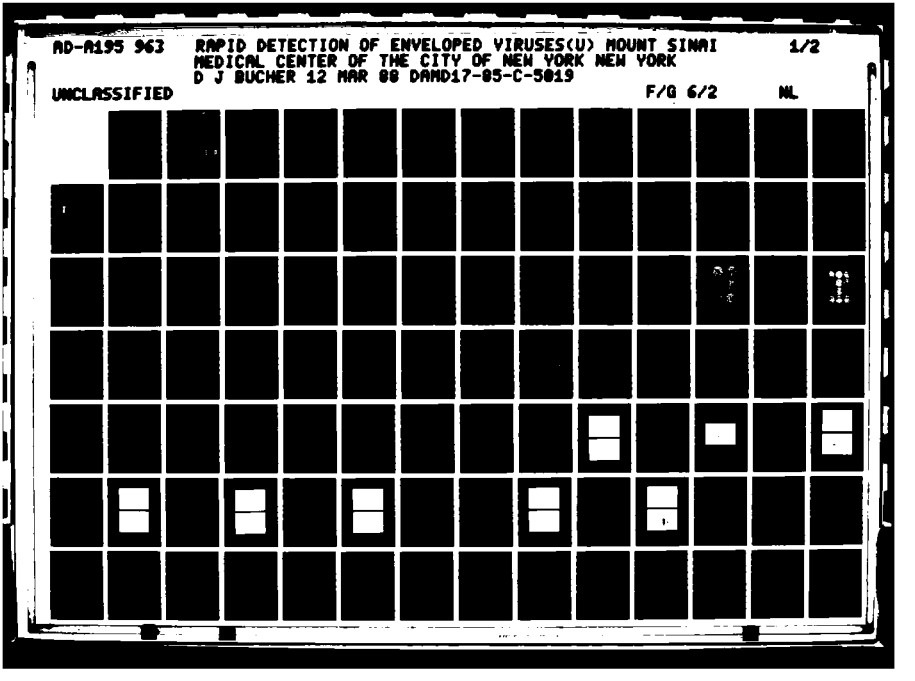

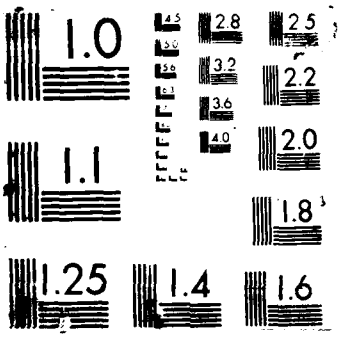
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RAPID DETECTION OF ENVELOPED VIRUSES

ANNUAL/FINAL REPORT

DORIS J. BUCHER, Ph.D.

March 12, 1988

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19. ABSTRACT (Continue on reverse if necessary and identify by block number)

M-protein of influenza virus is a type-specific antigen and the most invariant protein of the virus. A rapid virus detection system based on M-protein detection would detect all type A influenza viruses and be independent of antigenic shift and drift. To provide a source of highly specific antibodies to M-protein for viral detection, a panel of 18 hybridoma lines secreting monoclonal antibodies reactive with M-protein of type A influenza virus was developed. Specificity was confirmed by the Western blot technique. Titers of ascites fluids as assayed by ELISA were frequently in excess of 100,000.

Epitope analysis was performed by competitive analysis utilizing competition of unconjugated monoclonal antibodies with alkaline phosphatase-conjugated monoclonal antibodies on M-protein coated ELISA plates. A minimum of three antigenic sites were found with site 1 represented by 2BB10-C12 (G9 and A5), site 2 represented by 1G11-D11 (E3-F3) and 611-G10-D3 recognizing a third epitope. This data supports our findings that combinations of these antibodies provided maximal sensitivity in virus detection.

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Monoclonal antibodies in sites #2 and #3 reacted with a synthetic peptide representing residues 83-100 of M-protein.

.Generally, the monoclonal antibodies showed broad reactivity against a range of type A influenza viruses (12 strains representing 4 human and avian hemagglutinin subtypes). No reactivity was seen with type B influenza virus. A subset of antibodies in antigenic site #1 was found to react only with A/PR/8/34 and A/USSR/90/77. Broad reactivity was seen for two prototype monoclonal antibodies recognizing distinct antigenic sites, 2BB10-C12 (G9 and A5) and 1G11-D11(E3-F3). The narrowly reactive monoclonal antibodies provide a sensitive means of identifying the A/PR/8/34 M-protein associated with high yield in reassortant viruses developed for use in vaccines.

Monoclonal antibodies to M-protein from several hybridoma lines were found capable of detecting type A influenza viruses in 'seeded' clinical specimens to a level of 10 ng/100 ul. Analysis of a collection of H1N1 positive specimens showed higher reactivity than negative controls. Similar results were found with our collection of H3N2 specimens. Good reactivity was seen following amplification in ovo or in tissue culture, suggesting the use of M-monoclonal antibodies for rapid typing.

Influenza viruses (H3N2 and H1N1) could be directly detected in nasopharyngeal specimens utilizing monoclonal antibodies to M-protein in the TR FIA (time-resolved fluoroimmunoassay) system. Optimal sensitivity was achieved when the capture and detecting antibodies recognized different antigenic sites of M-protein. Sensitivity was up to seventy fold above background levels.

Immunofluorescence experiments utilizing the M-protein monoclonal antibodies in virus-infected cells demonstrated that (1) M-protein enters the nucleus during the viral replicative cycle and (2) M-protein binds to actin filaments in infected cells. The use of a vaccinia recombinant containing the M-protein gene demonstrates that the signal for nuclear transport of M-protein is located within the M-protein sequence; synthesis of other viral proteins is not necessary for nuclear transport of M-protein.

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Summary

Much of our effort during the period of this contract was devoted to characterization and expansion of our panel of monoclonal antibodies to M–protein of Type A influenza virus. At this time we have a total of 18 distinct hybridoma lines secreting monoclonal antibodies reactive with M–protein. Ascites pools were generated for all of these lines and the monoclonal antibodies purified by gel chromatography. All monoclonal antibodies shown to react with M–protein by microtiter ELISA were also shown to be reactive by the Western blot technique.

The monoclonal antibodies were titered against a broad range of type A influenza viruses (12 strains). Broad reactivity was observed for the majority of the monoclonal antibodies. A subset of antibodies was found which were reactive only with A/PR/8/34 M–protein within antigenic site #1 as typified by 2E5–C1 except for the peculiar reactivity of M–protein from A/USSR/90/77. Broad reactivity is important for the development of a 'universal' type A influenza virus detection system. Prototype monoclonal antibodies recognizing two distinct antigenic sites on M–protein, 2BB10–C12 and 1G11–D11, selected earlier for their unusual avidity and sensitivity for virus detection showed consistently high levels of reactivity across all strains and subtypes. Any variation seen in titer for these two monoclonal antibodies among viral strains appeared to be more likely due to degree of purity of virus preparation than differences in absolute reactivity with M–protein. The narrowly reactive monoclonal antibodies were also shown to have an important practical application; they were capable of detecting the A/PR/8/34 M–protein in a reassortant virus which is a candidate virus for influenza vaccine production. The A/PR/8/34 M–protein has been associated with high yield of virus in ovo.

Combinations of purified monoclonal antibodies to M–protein used as capture antibodies for type A influenza viruses were superior to the use of a single monoclonal antibody for virus capture. The combination which produced the most sensitive level of virus detection was 2B–B10–C12 and 611–G10–D3. 1G11–D11 and 2B–B10–C12 also provided sensitive detection for X–53a (H1N1) virus although this combination did not provide sensitive detection of 15–2 (H3N2) virus.

These results correlate with our findings on epitope analysis of the monoclonal antibodies to M–protein. Results from competitive inhibition of alkaline phosphase conjugated monclonal antibodies with unconjugated monoclonal antibodies suggest that 2B–B10–C12 (G9 and A5) and 1G8–All are directed against one eptitope, 611–G10–D3 is directed against a second epitope and 1G11–D11 (1G11–E3–F3) interacts with a third epitope. Therefore, it would be predicted that combinations of monoclonal antibodies recognizing different epitopes would provide maximal sensitivity. Additional analyses performed with competitive inhibition assay permitted placement of the majority of our fourteen distinct monoclonal antibodies into three antigenic sites. These results were further corroborated by our TR FIA analyses showing maximum sensitivity when monclonal antibodies from different antigenic sites served as capture and detecting antibodies.

Antigenic sites #2 and #3 were localized to the M–protein region represented by synthetic peptide #2, residues 83–100 of M–protein. By deduction, based on the PR/8/34 specific nature of the 2E5–C1 monoclonal antibody and comparison of known sequences of M–protein from different strains we have localized antigenic site #1 to regions surrounding either residues #116 or #231.

A number of parameters in the ELISA test system were explored to optimize conditions to obtain increased sensitivity of virus detection through the use of monoclonal antibodies to M–protein. Monoclonal antibodies and rabbit polyclonal antibodies required purification from ascites fluid or sera for use in the capture assay system. A column chromatographic technique was developed for purification of the antibodies. Based on the results of variation of parameters of our capture assay system, a combination of purified monoclonal antibodies, 2B–B10–C12 and 1G8–All, were originally used to coat the microtiter plates for use as capture antibodies. More recent work with combinations of monoclonal antibodies suggest a better combination is 2B–B10–C12 (or G–9) and 611–G10–D3; this combination was used as the capture phase for analysis of specimens from the 1986–1987 winter influenza season. Monoclonal antibodies to M–protein from several of our hybridoma cell lines have been found capable of detecting virus in artificially seeded clinical specimens down to levels of about 10 ng in 100 μl.

Analysis of clinical specimens by ELISA showed a distinctly higher reactivity with a collection of H1N1 positive specimens obtained from Dr. Meikeljohn's laboratory as compared with negative controls. Earlier results with our own collection of H3N2 specimens (1984–85) also showed a higher degree of reactivity as compared with controls. Results with both collections support the validity of use of M–protein capture monoclonal antibodies for detection of type A influenza viruses.

Good ELISA reactivity was seen following amplification of 1984–85 H3N2 positive specimens in eggs suggesting the use of the M–capture system for typing influenza viruses. Considering the vagaries of the hemagglutination inhibition test, the M–capture ELISA system may be highly useful in typing influenza isolates. However, H3N2 specimens from the 1985–86 influenza season amplified in MDCK cells have dramatically lower reactivity than observed for H3N2 isolates made in the 1984–85 winter. Further investigation is necessary to determine if the epitope of M–protein reactive with the monoclonal antibodies has mutated (or is the result of a reassortment event) in the H3N2 influenza viruses circulating in 1986. Greater sensitivity was achieved with tissue culture amplified specimens and ELISA assay with 1G11–D11. The same monoclonal antibody, 1G11–D11, which served well in TR FIA also was the most sensitive capture antibody for ELISA. No detergent was used at the initial step of exposure of the antibody coated plate to specimen; 1G11–D11 was found to be very unstable in the presence of detergents.

Direct detection of type A influenza viruses (H1N1 and H3N2) in nasopharyngeal specimens was achieved in a time resolved fluorimmunoassay system (TR FIA). This assay required the use of monoclonal antibody reactive with one epitope of M–protein as capture antibody and monoclonal antibody to a second epitope (Europium chelated) as the detecting antibody. Optimal sensitivity was achieved when the capture and detecting antibodies used recognized different epitopes of the M–protein antigen. Sensitivity was up to seventy fold above background levels; specimens which had been shown to be positive for type B influenza viruses by ELISA testing were at a background level.

Immunofluorescent visualization of M–protein during the replicative cycle has demonstrated that (1) M–protein enters the nucleus during viral infection and (2) M–protein binds to actin filaments during the replicative cycle. Investigators had reported differing results as to whether or not M–protein entered the nucleus during influenza virus replication. Interaction of M–protein and actin had been reported for Newcastle Disease virus, a paramyxovirus, using purified proteins; our experiments are the first to show interaction of M–protein and actin directly in the cell. The use of a

vaccinia recombinant containing the M–protein gene demonstrates that the signal for M–protein transport to the nucleus is located within M–protein; synthesis of other influenza viral proteins is not necessary for nuclear transport of M–protein to occur.

Our collaborative work with Dr. Judd at S.R.I. on the production of immunoreactive peptide segments of M–protein is important to this project from at least two aspects. Important applications for these findings are: (1) Synthetic peptide segments of M–protein can be used in ELISA assays as adsorbents to determine the precise epitope seen by the monoclonal antibodies to M–protein. (2) Synthetic peptides of regions of M–protein may serve as adsorbent antigen in place of M–protein in viral detection systems based on competitive inhibition assay. (3) Synthetic peptides to M–protein may be valuable as components in a vaccine to protect against influenza. Three peptides have been synthesized and analyzed with a panel of rabbit antisera to several subtypes of influenza virus. These peptides have been shown to be immunoreactive in that they react with preformed antibody to influenza virus with median and mean fold increases of 60.8 and 8.0, respectively, for peptide #1;28.1 and 4.2 for peptide #2; and 12.9 and 4.3, respectively, for peptide #3 when preimmune and hyperimmune sera are compared.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86–23, Revised1985).

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I. Hybridoma Lines Secreting Monoclonal Antibodies to M–protein Influenza Virus (Type A)

During the course of this contract, a total of eighteen hybridoma lines (and their subclones) secreting monoclonal antibodies to influenza virus M–protein were developed and/or characterized (see Table I). The first seven lines listed in Table I were developed prior to the initiation of the contract period; the additional 11 lines were developed through Feb. 15, 1988.

The hybridoma lines were developed by fusion of spleen cells from Balb/c mice immunized with purified M–protein with myeloma cells (Sp2/0–Ag14). The protocol is described in the Appendix. M–protein was purified by SDS gel chromatography from outdated swine influenza vaccine donated by the Canadian Bureau of Biologicals. This vaccine was prepared from the X–53a strain of influenza virus (a reassortant containing the A/PR/8/34 M–protein gene)[1,2]. Hybridomas secreting monoclonal antibodies reactive with M–protein were selected through analysis of supernatants from tissue culture wells by ELISA utilizing microtiter plates coated with purified M–protein (40 ng/well) and virus (X–53a strain, 100 ng/well)[2]. Sendai virus and serum albumin coated wells were included in the ELISA assay as controls to permit elimination of those hybridomas secreting monoclonal antibodies reactive with host antigen or of low specificity.

Hybridoma lines secreting monoclonal antibodies reactive with M–protein were subcloned (see Appendix). The isotypes of monoclonal antibodies were determined utilizing the mouse immunoglobulin subtype identification kit (Boehringer–Mannheim). The majority of the monoclonal antibodies were found to be of the IgG1 isotype; all monoclonal antibodies were found to have kappa light chains. Cloned hybridomas were amplified and stored in liquid nitrogen following a period of slow freezing in the Revco (-70°C.). Hybridomas were frozen according to the protocol described in the Appendix. In order to obtain the maximal antibody concentration possible, ascites fluids were developed for all hybridoma lines in pristine–primed Balb/c mice (see Appendix). Approximately 40–50 ml of ascites fluids were obtained by serial propagation of the hybridoma cells in mice.

A. Maintenance of Hybridoma Collection

A number of factors were examined with respect to conditions for freezing of our hybridoma lines for liquid N_2 storage as well as the method for thawing out and recovery of viable cells once frozen.

1. Use of high quality dimethylsulfoxide (DMSO)

A number of our hybridoma lines had been frozen utilizing laboratory grade DMSO which had been sitting on the shelf for several years. Our recovery rate of viable clones on thawing was very low (one in five or six clones). We now use DMSO stored under nitrogen in small ampules and ordered in small quantities (Sigma). The use of higher quality DMSO (10% concentration) resulted in a two– to three– fold increase in recovery rate of viable clones on freezing and thawing.

Table 1

HYBRIDOMA LINES SECRETING ANTIBODIES TO M-PROTEIN

	ISOTYPE*	ELISA titers ($X10^{-3}$) M-PROTEIN	X-53a	RATIO
1. 2B-B10-C12	IgG_1	5200	4300	1.2
2B-B10-F1	IgG_1	2200	1500	1.5
2B-B10-A5	IgG_1	498	186	2.7
2B-B10-G9				
2. 2E5-C1	IgG_1	5475	3900	1.4
2E5-D4	IgG_1	4933	1917	2.6
3. 6B9-B8	IgG_1	4850	48	101.0
6B9-G3	IgG_1	5800	11	527.3
4. 3G12-C12	IgG_1	5800	1600	3.6
5. 9E8-B2	IgG_1	280	120	2.3
6. 1G11-D11	IgG_1	3749	729	5.1
1G11-E3-F3				
7. 1G8-A11	IgG_1	2187	647	3.4
1G8-H9	IgG_1	609	336	1.8
8. 611-G10-D3	IgM	243	59	4.1
9. 611-B12-D10	IgG_{2a}	207	101	2.0
10. 951-C4-G2	IgG_1	712	522	1.4
11. 963-D3-G10	IgG_1	645	649	1.0
12. 961-G8-H3	IgG_1	564	400	1.4
13. 951-D10-B3	IgG_{2b}	1344	57	23.6
14. 961/6-B10	IgG_1	354	2	177.0
15. 823-D8-B11	IgG_1			
16. 833-G9-H9	IgG_1			
17. 833-C3-A9	IgG_1			
18. 851-D2-D11	IgG_1			

2. Rate of addition of DMSO

We have also found that slowing the rate of addition of DMSO, prior to freezing down cells, or the rate of dilution from DMSO on thawing cells after liquid nitrogen storage apparently enhances recovery of viable cells. Our protocol for freezing cells following pelleting now involves a two–stage addition of DMSO over 5–10 minutes. Cells are brought to a 5% DMSO concentration in media, allowed to sit for 5 minutes, then adjusted to a final concentration of 10% DMSO before overnight slow cooling in the Revco prior to storage in liquid N_2. On thawing, cells are rapidly thawed at 37°C followed by addition of medium to decrease to concentration to 5%, a 5 minute wait, followed by addition of medium to dilute DMSO to 2.0%. Cells are either incubated directly in DMSO overnight at 37°C followed by change of the medium the next day or centrifuged and resuspended in medium after dilution to 2.0%.

3. Addition of Thymocytes

The addition of thymocytes greatly enhanced the rate of recovery of recalcitrant clones. The thymus was removed from young mice and minced to liberate T–cells. The T–cells were added to a concentration of 1–2 X 10^6/mL of medium containing hybridoma cells. Up to 10 times the number of B–cells was seen the following day after the addition of T–cell as compared with the same sample which had not received T–cells. In addition, some hybridoma lines with very low number of viable hybridoma cells several days following thawing T–cells could be revived if T–cells were added to the media.

B. Western Blot Analysis

A protocol was developed by Steve Popple for analysis of monoclonal antibodies by the Western blot technique to verify their specificity for M–protein (see Appendix). This technique provides an alternative method in addition to ELISA analysis (following coating of polystyrene plates with M–protein) for detection of monoclonal antibodies reactive with M–protein.

Western blot analysis involves the transfer of proteins to nitrocellulose membranes following their separation by SDS slab gel electrophoresis. Electrophoretic transfer was employed for this procedure. The monoclonal antibodies undergoing analysis are allowed to interact with the 'blotted' membrane following 'blocking' with the appropriate BSA–serum mixture. Binding of monoclonal antibody is detected by adding alkaline–phosphatase conjugated sheep (or goat) anti–mouse antibodies and incubating with a substrate (Promega Biotec) which precipitates on cleavage forming an insoluble blue precipitate at the site of enzyme activity and antibody localization. A detailed protocol is provided in the appendix.

Quantities as low as one microgram total viral protein or 0.5 microgram M–protein were sufficient for electrophoresis, transfer and detection. All monoclonal antibodies which were previously determined to have M–protein specificity by the ELISA assay were confirmed as M–protein specific by the Western blot technique. (See figure 1)

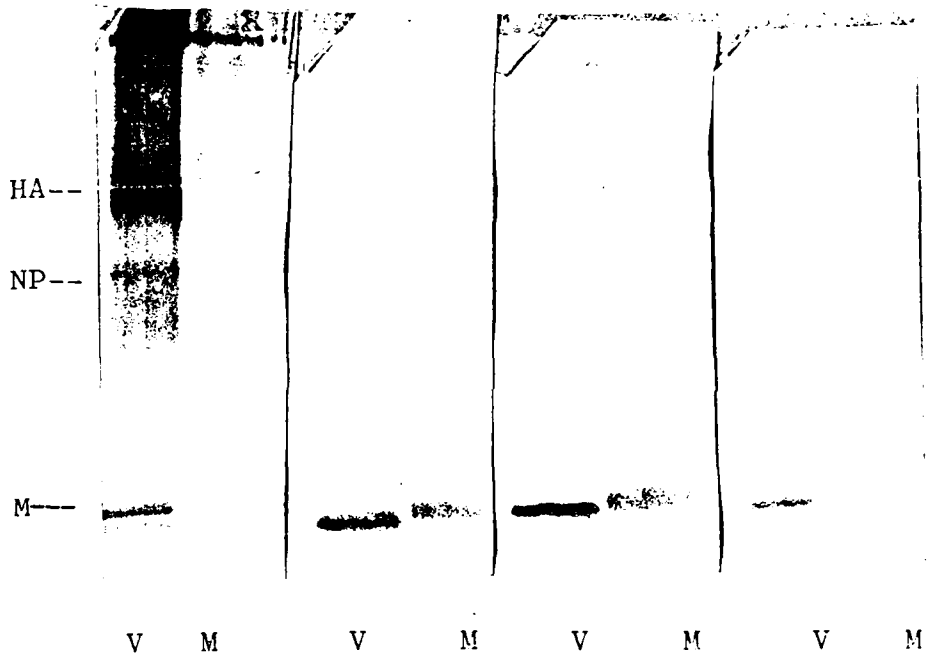


Figure 1. Western blot analysis of total X-53a (H1N1) virus (V) or M-protein (M) (non-reduced) following electrophoresis and electrophoretic transfer. Nitrocellulose strips were incubated *with the following antisera or ascites preparations;* anti-PR-8 (H1N1) virus or hybridoma lines directed against M-protein, 2B-B10-G9, 1G11-D11 and 611-G10-D3 developed according to the Western Blot protocol (see appendix).

C. Purification of Monoclonal Antibodies for Use in a Capture System

In screening ascites fluids from a number of hybridoma lines for their ability to capture M–protein and virus, it was found that in only a few rare instances could crude ascites fluids be used directly on ELISA plates as adsorbent. For most 'lines', it was necessary to purify the monoclonal antibodies to permit optimal uptake by the microtiter plates and provide maximal capturing ability.

Several approaches to purification of antibodies from ascites fluids were attempted. Purification by ammonium sulfate fractionation resulted in considerable variation in both percentage recovery and purity of preparations for ascites fluids from different hybridoma lines or for IgG as compared with IgM.

We had routinely isolated antibodies from serum using QAE–Spehadex chromatography in this laboratory. Analysis of proteins recovered and percentage recovery also showed considerable variation in the product.

Since most of our monoclonal antibodies are from the IgG1 subclass, they will not bind to staphylococcal protein A. Therefore, protein A chromatography was not attempted.

Gel chromatography using LKB ultrogel ACA–34 (range 20,000–350,000) was found to be the optimal choice for purification of IgG monoclonal antibodies. ACA–22 has a slightly larger pore size and was utilized for purification of IgM. Chromatography of ascites fluid (following centrifugation at 1,500 rpm for 15 minutes) was performed on either a 2x5 X 90 cm column (2 to 4 mL ascites fluid or serum) or a 5.0 X 70 cm column (6 to 10 mL ascites fluid or serum).

Chromatography was performed at room temperature in phosphate buffered saline (pH 7.0) with the addition of 0.5% sodium azide. Absorbance (260 nm) of effluent was continously monitored using a flow cell and recorder. The IgG fraction was well separated from serum albumin by this technique (see Figure 2). Gel chromatography permits close to 100% recovery IgG regardless of subclass. Protein concentration was determined by measuring the absorbance of the pooled IgG at 280 nm. An extinction coefficient of E_{280} nm (1% IgG) = 13.8 O.D.U. was used to convert absorbance to mg/mL.[3]

D. Antigenic Site Analysis

1. Conjugation of purified monoclonal antibodies with alkaline phosphatase

Monoclonal antibodies were purified by column chromatography and conjugated with alkaline phosphatase accroding to the protocol of Voller and associates[4] and Kearney and coworkers[5]. (see protocol in Appendix). Some monoclonal antibodies efficiently conjugated, others were less effectively conjugated and a third group was nearly refactory to conjugation. (see Table 2). The monoclonal antibody which could not be conjugated with alkaline phosphatase, 611–B12–D10, was of the IgG_{2a} isotype, the other monoclonal antibodies were IgG_1 or IgM. All had kappa light chains. Conjugation efficiency is dependent on the availability of the appropriate amino acids for linkage with glutaraldehyde. Specific activities obtained are also dependent on the absolute purity of the antibody preparation. Conjugation of polyclonal antibodies

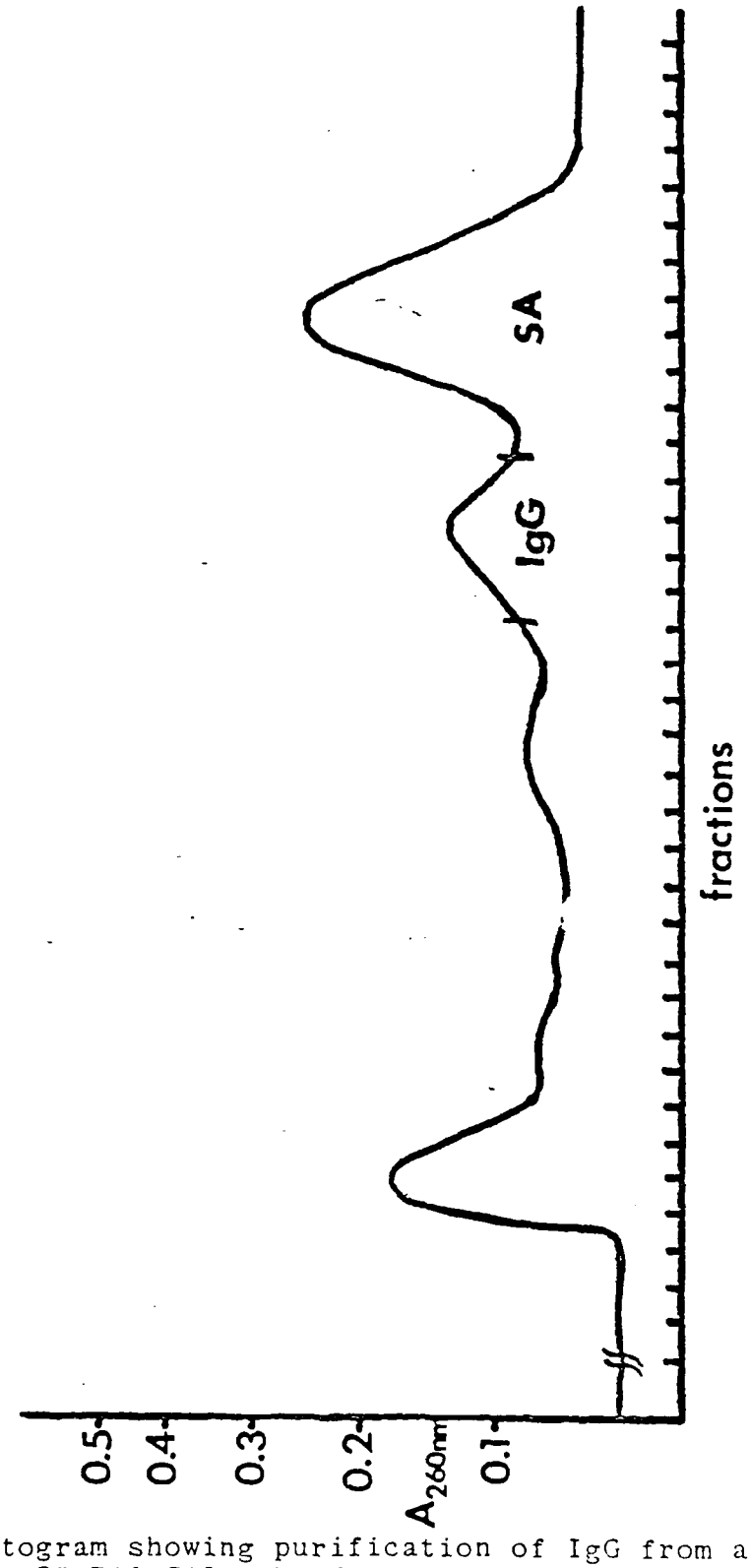


Figure 2. Chromatogram showing purification of IgG from ascites fluid produced for the 2B-B10-C12 hybridoma clone. Ascites fluid (4 mL) was chromatographed on an ACA-34 column (2.5 X 90 cm) in phosphate buffered saline containing 0.05% sodium azide.

Table 2

Alkaline Phosphatase-Conjugated Monoclonal Antibodies

Conjugated MAb	mg/ml	ELISA ASSAY Titer (3 fold above bkgd)	
		M-protein	whole virus (X-53a)
2B-B10-C12	0.36	15,800	3600
2B-B10-G9	0.50	3,000	770
2B-B10-A5	0.50	5,000	2000
1G8-A11	0.44	20,500	3500
1G11-D11	0.44	10,000	5100
611-G10-D3	0.50	3,000	460
611-B12-D10 (IgG_{2a})	0.40	-	-
M - antisera - rabbit polyclonal	0.71	550	830

resulted in a rather low titer. This probably reflects the fact that only a small proportion of the antibodies even in hyperimmune sera is targeted toward M–protein. Affinity column isolation (with M–protein columns) of antibodies from the polyclonal serum would probably increase specific activity.

2. Antigenic Site Analysis

Competitive inhibition analyses were performed between alkaline–phosphatase conjugated and non–conjugated monoclonal antibodies by a 'blocking' ELISA assay. The assay was performed following a two–fold dilution of duplicate of unconjugated monoclonal antibody (1 μg in 200 μl PBS–Tween) from row 2 of a 96 well microtiter plate coated with 40 ng/well M–protein. Following incubation and washing steps, monoclonal antibody conjugated with alkaline phosphatase was added at a sufficient concentration to generate 0.4–0.8 absorbance units after one hour incubation. This generally required a dilution of 1:500 to 1:3000 of the conjugated monoclonal antibody. Results of competitive inhibition analyses with 2B–B10–G9 as the conjugated antibody are shown in figure 3; 1G11–D11 and 611–G10–D3 as the conjugated antibody are shown in figures 4 and 5.

Results from competitive inhibition suggest that 1G8–A11 and 2B–B10 (C–12, G9, A5) share the same antigenic site since 1G8–All is capable of nearly complete blocking of 2B–B10 lines and the converse also holds. Monoclonal antibodies which did not block or only partially blocked include 611–G10–D3, 611–B12–D10, and 1G11–D11. Earlier lines including 3G12–C12 and 9E8–G10 totally blocked 2B–B10–C12 and 1G8–All and therefore share the same antigenic site. 2E5–C1 shows partial blockage at a low level and therefore must occupy a site near to that of 2B–B10–C12 but slightly separated.

Based on these results, 611–G10–D3 is a good choice for use with 2B–B10–C12 (G9 or A5) to maximize coverage of antigenic sites and produce maximal sensitivity for virus dettection. This was also found in practice when combinations were made for virus detection. However, antigenic sites analysis may be difficult to interpret since 611–G10–D3 is an IgM monoclonal antibody. 611–B12–D10 should also be a good choice based on antigenic site analysis; it shows no blocking activity with 2B–B10–C12 or 1G8–All. However, in practice, 611–B12–D10 functioned poorly as a partner of either 2B–B10–C12 or 1G8–All.

1G11–D11 also showed only slight blocking of 2B–B10–C12 or 1G8–All suggesting that 1G11–D11 views a different antigenic site from 2B–B10–C12 or 1G8–All. Blocking experiments with 1G11–D11 as the conjugated monoclonal antibody further suggest that the antigenic site seen by 1G11–D11 is different from that of 611–G10–D3 and that this may be a third site. Experiments with combinations of 1G11–D11 and 611–G10–D3 did not show enhanced viral capture activity. However, 1G11–D11 did complement 2B–B10–C12 well in increasing viral capture sensitivity for X–53a (H1N1), but with less success versus 15–2 (H3N2).

Therefore to date, we have identified three distinct antigenic sites on M–protein. Site one includes 2B–B10 (C12, G9, A5), 1G8–All, 3G12–C12, and 9EG–G10; 2E5–C1 is in the site one region but slightly removed. Site 2 includes G11–D11 and Site 3 includes 611–G10–D3. However, 611–G10–D3 is an IgM molecule and may cause problems interpreting competition data. The results of antigenic site analyses are summarized in Table 3.

FIGURE 3

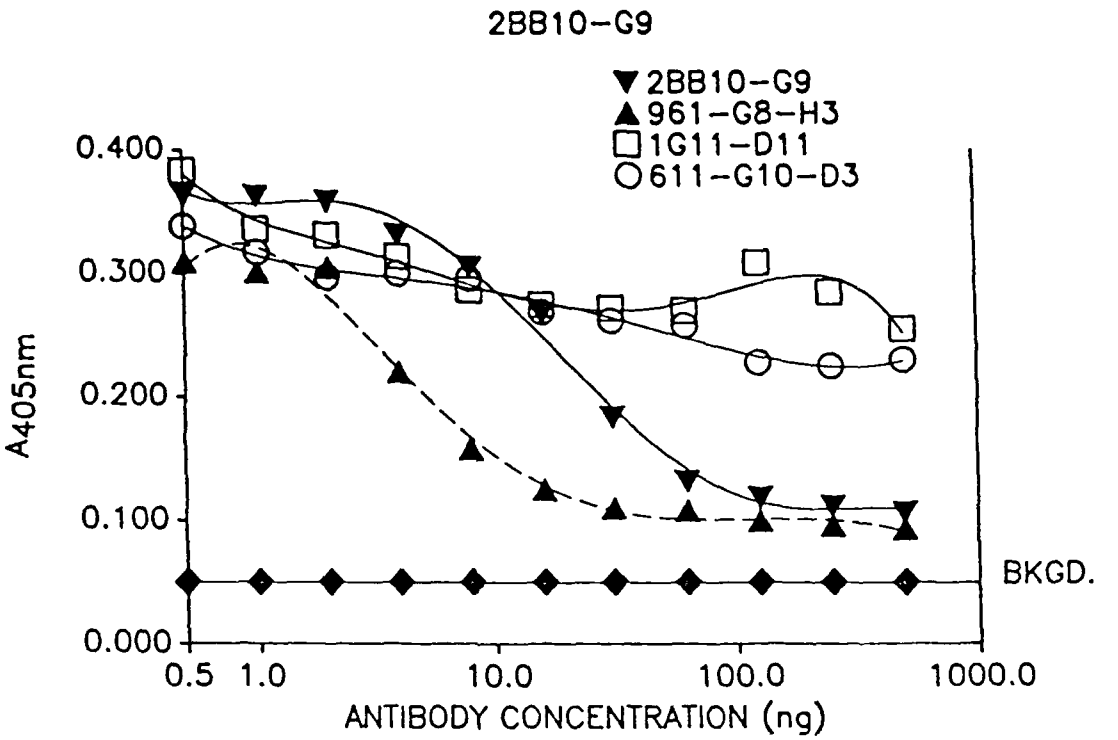


Figure 3. Competitive analysis was performed by diluting out the monoclonal antibody across the twelve wells of a microtiter plate. The wells were previously coated with 40 ng M-protein. Blocking activity of the monoclonal antibody was observed against 2BB10-G9 monoclonal antibody conjugated with alkaline phosphatase and diluted to provide an enzyme activity capable of cleaving p-nitrophenyl substrate in one hour to yield approximately 0.4 $A_{405\ nm}$.

FIGURE 4

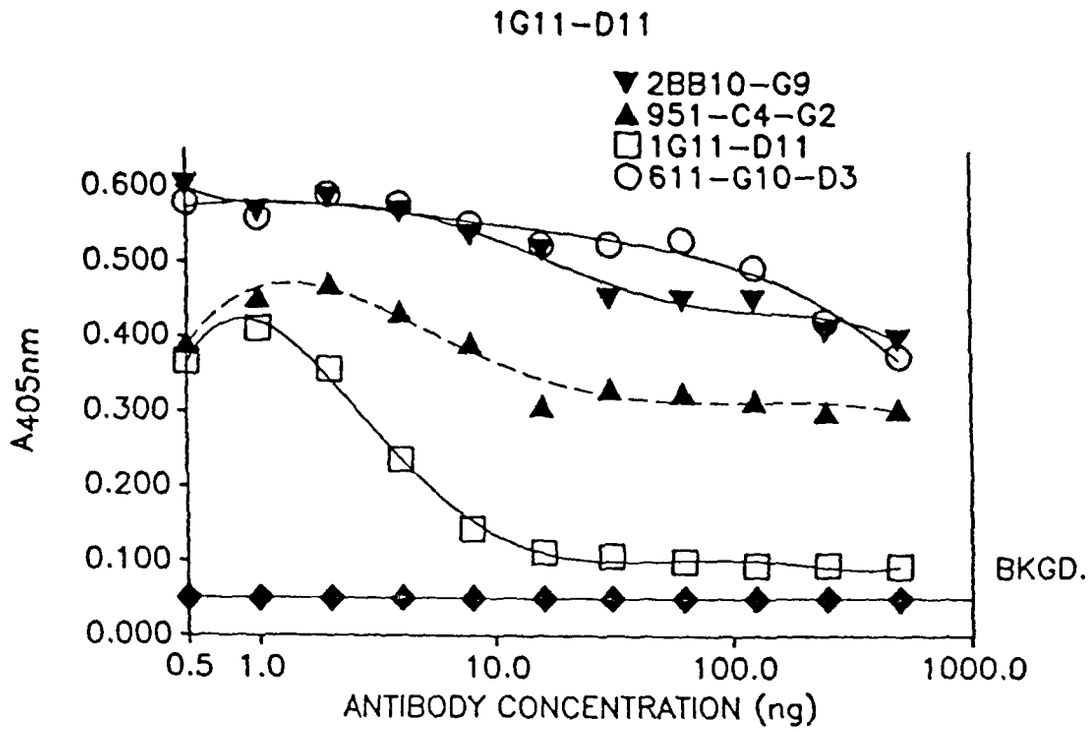


Figure 4. See figure 3. 1G11-D11 alkaline phosphatase conjugate used.

FIGURE 5.

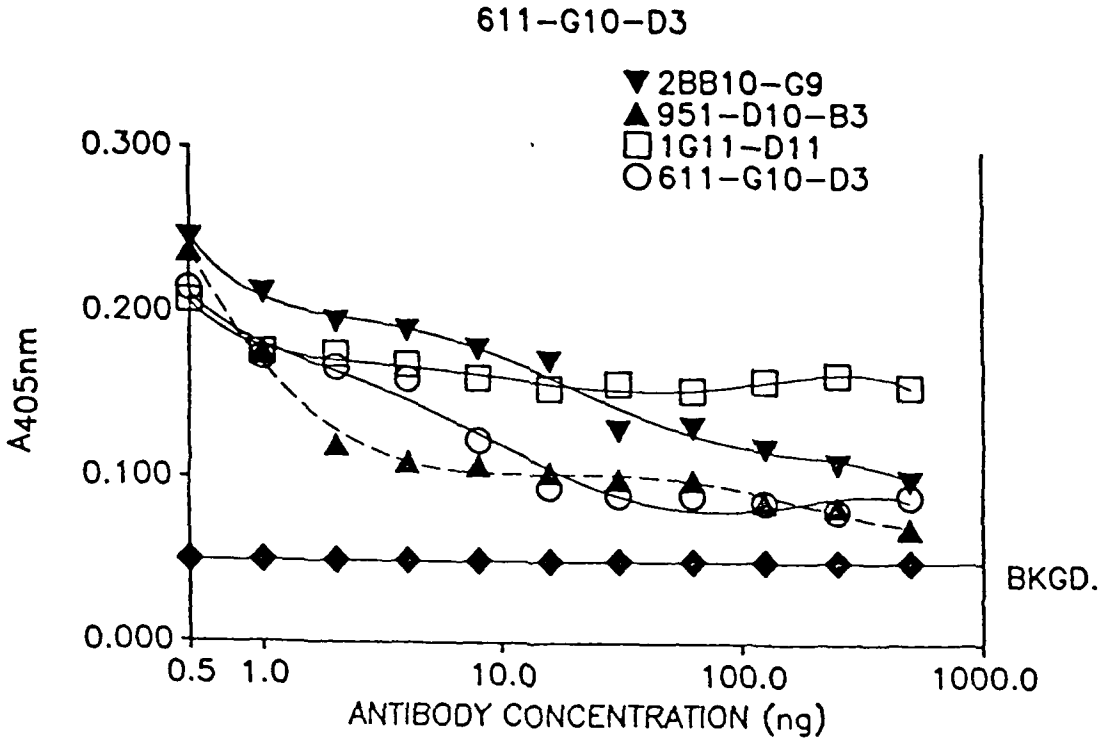


Figure 5. See Figure 3. 611-G10-D3 alkaline phosphatase conjugate used.

TABLE 3

MONOCLONAL ANTIBODIES REACTIVE WITH
INFLUENZA A M-PROTEIN:
CLASSIFICATION INTO ANTIGENIC SITES

Antigenic Site #1	Antigenic Site #2	Unclassified
2BB10-C12,F1,A5,G9	1G11-D11	611-B12-D10
2E5-C1,D4	951-C4-G2	961/6-B10
6B9-B8,G3	(M2-1C6)*	(904/6)*
1G8-A11,H9	(289/1)*	
3G12-C12	Antigenic Site #3	
9E8-B2	611-G10-D3	
963-D3-G10	951-D10-B3	
961-G8-H3		

*Described by van Wyke and coworkers (1984) J.Virol. 49:248.

C. Reactivity of Monoclonal Antibodies with a Broad Panel of Influenza Viruses

The monoclonal antibodies to M–protein were tested for their reactivity with a broad panel of type A influenza viruses representing human and avian strains from the H1N1, H2N2, H3N2, and H3N8 subtypes. (see Table 4) The panel includes the six strains for which M–protein sequence information is available. Influenza virus strains were inoculated into monolayers of MDCK cells, the cells were harvested and the virus preparations purified on sucrose gradients. Protein concentrations were determined with the Lowry assay.

ELISA was performed following coating overnight of each well with 100ng influenza virus in carbonate buffer. The wells were postcoated, washed and 100μl ascites fluid diluted in 3–fold increments. The plates were washed and sheep anti–mouse IgG conjugate added. Substrate was added following a washing step. The plates were allowed to develop and endpoints calculated as the dilution which was threefold above the background value.

Five of the monoclonal antibodies assigned to antigenic site #1 showed broad reactivity with high titer for all 12 type A strains tested. These included 2BB10–G9, 1G8–A11, 3G12–C12, 9E8–B2 and 821–B8–A8. However, a subset within antigenic site #1 was highly specific for A/PR/8/34 (and A/USSR/90/77). These monoclonal antibodies, 2E5–C1, 961–G8–H3 and 963–D3–G10 had titers in the range of 200,000 to 800,000 for viruses containing A/PR/8/34 M–protein (A/PR/8/34 and X–53a); other viruses had titers of less than 300 for 2E5–C1 and slightly higher values for 961–G8–H3 and 963–D3–G10. An interesting exception to the above was A/USSR/90/77. This virus, reappearing in 1977, shares the 2E5–C1 reactive epitope with A/PR/8/34. Its ractivity profile with other monoclonal antibodies to M–protein is quite similar to A/PR/8/34 with the exception of four–fold lower reactivity for M2–1C6 and 18–fold lower reactivity with 611–B12–D10.

Monoclonal antibodies to antigenic site #2 generally react broadly with all virus trains tested. 951–C4–G2 shows the greatest variation in titer with values of 300,000–500,000 versus A/PR/8/34 M–protein containing viruses and only 23,000 versus A/WSN/33. Although A/PR/8/34 and A/WSN/33 were isolated one year apart, they show considerable differences in their reactivity profiles with the monoclonal antibodies to M–protein.

Antigenic site #3 comprised of 611–G10–D3 and 951–D10–B3 does not show broad reactivity. Values range from 160,000 to <300. 951–D10–B3 displays an eight–fold higher titer with A/Texas/1/77 and a six–fold higher titer with A/Pt.Chalmers/1/73 than with the A/PR/8/34 M–protein to which it was originally developed. A mutation in this site evidently occurred for M–protein of the A/Bangkok/1/79 strain resulting in loss of reactivity. An earlier mutation in the H3N2 M–protein may have occurred to generate the reactive site in A/Pt.Chalmers/1/73; no reactivity was found with A/Udorn/307/72.

The monoclonal antibodies originally selected for their high titer and broad reactivity, 2BB10–G9 and 1G11–D11, based on testing of a limited panel of virus strains have been found to be the optimal monoclonal antibodies for detection of a broad range of virus strains. Neither 611–G10–D3 nor 951–D10–B3 from antigenic site #3 show broad reactivity.

Table 4. Reactivity of Monoclonal Antibodies with Type A Influenza Viruses

ELISA TITERS $X10^{-3}$

	M protein (purified)	H1N1				H2N2	
		X-53a	A/WSN/33	A/PR/8/34	A/USSR/90/77	A/Sing/1/57	A/Mallard/NY/6750/78
Antigenic Site #1							
2BB10-G9	1839	695	318	708	465	1168	401
1G8-A11	631	292	526	376	363	477	187
3G12-C12	656	243	449	308	194	434	321
9E8-B2	195	148	473	164	340	193	194
821-B8-A8	N.D.	N.D.	500	N.D.	131	N.D.	101
2E5-C1	1468	796	< 0.3	784	1159	< 0.3	< 0.3
961-G8-H3	564	400	2	279	216	1.4	0.9
963-D3-G10	645	649	< 0.3	611	195	< 0.3	< 0.3
6B9-B8	53	1.6	< 0.3	0.5	0.8	< 0.3	< 0.3
Antigenic Site #2							
1G11-D11	2001	1271	417	1436	1278	1891	626
951-C4-G2	712	522	23	311	187	395	145
823-D8-B11	N.D.	N.D.	< 0.3	N.D.	260	N.D.	168
M2-1C-6**	459	95	23	119	28	N.D.	24
289/4-D5**	N.D.	N.D.	40	N.D.	14	N.D.	22
Antigenic Site #3							
611-G10-D3	79	4	0.4	3	0.8	22	< 0.3
951-D10-B3	1344	57	0.7	21	6	54	0.7
Unclassified							
6H-B12-D10	1737	114	63	55	3	49	11
961/6-B10	354	2	0.8	2	3	0.6	0.3

**Described by van Wyke and coworkers (1984) J. Virol. 49:248.

Table 4 (cont.)

	H3N2					H3N8	B/Le
	A/Aichi/2/68	A/Udorn/307/72	A/Pt.Chalmers/1/73	A/Texas/1/77	A/Bangkok/1/79	A/Duck/Ukraine/1/63	
Antigenic Site #1							
2BB10-G9	196	216	1063	670	192	208	<0
1G8-A11	196	149	531	527	271	204	<0
3G12-C12	215	106	284	526	224	183	<0
9E8-B2	200	240	188	161	430	198	<0
821-B8-A8	162	125	N.D.	N.D.	65	42	<0
2E5-C1	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	<0
961-G8-H3	2	0.7	2.2	3.5	0.9	0.8	<0
963-D3-G10	< 0.3	< 0.3	0.5	25	< 0.3	< 0.3	<0
6B9-B8	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	<0
Antigenic Site #2							
1G11-D11	427	484	1180	1005	473	223	<0
951-C4-G2	66	92	461	506	133	95	<0
823-D8-B11	196	205	N.D.	N.D.	168	109	<0
M2-1C-6**	21	39	N.D.	N.D.	22	16	<0
289/4-D5**	15	7	N.D.	N.D.	6	15	<0
Antigenic Site #3							
611-G10-D3	< 0.3	< 0.3	15	5	< 0.3	< 0.3	<0
951-D10-B3	0.9	< 0.3	118	160	< 0.3	0.4	<0
Unclassified							
611-B12-D10	23	15	63	177	16	7	<0
961/6-B10	0.7	0.4	0.8	0.8	0.3	< 0.3	<0

**Described by van Wyke and coworkers (1984) J. Virol. 49:248.

D. Determination of Amino Acid Sequences of Antigenic Sites Recognized by Monoclonal Antibodies to M–protein.

Synthetic peptides had been prepared to regions of M–protein by Dr. Amrit Judd of S.R.I. International. Dr. Judd selected segments to synthesize based on her analysis of hydropathicity and secondary structure (of M–protein). Sequences were selected within hydrophilic domains that included hydrophilic and hydrophobic domains where the protein might be exptected to turn and expose 'corners'. These synthetic peptides represent the following segments of A/PR/8/34 M–protein: peptide #1, 66–78; peptide #2, 83–100; and peptide #3, 152–166. Sequences of the peptides are as follows:

Peptide No.

1. H–Leu – Thr – Val – Pro – Ser – Glu – Arg –

   Gly – Leu – Glu – Arg – Arg – Arg – OH

2. H–Ala – Leu – Asn – Gly – Asn – Gly – Asp – Pro –

   Asn – Asn – Met – Asp – Lys – Ala –

   Val – Lys – Leu – Tyr – OH

3. H–Glu – Gln – Ile – Ala – Asp – Ser – Gln –

   His – Arg – Ser – His – Arg – Gln – Met –

   Val – OH

In earlier analyses we were unable to demonstrate any reactivity of the peptides with the monoclonal antibodies. However, we decided to re–examine the interaction of peptides with monoclonal antibodies utlizing both peptides and peptide conjugates as immunoadsorbents. Earlier analyses utilized overnight incubations of ascites with peptide–coated plates resulting in high background; incubation times were reduced to one hr. for the present series of experiments. Immulon 2 plates (Dynatech) were coated with M–peptide (1/μg/well) or M–protein (0.040 μg/well) in 0.1 ml carbonate coating buffer for 18 hr. Following washing of the plates and postcoating, ascites fluids were diluted out in a three fold dilution series beginning with a 1:30 dilution. The plates were incubated one hour following washing of the plates, sheep anti–mouse IgG conjugate was added and the plates incubated an additional hour. The plates were washed and substrate (p–nitrophenylphosphate) was added. The plates were allowed to incubate for 1/2 hour and the results read on the ELISA plate reader (Titertek Multiscan MC). Endpoint titers were calculated as the dilution which had an absorbance three times above background absorbance. The results are presented in Table 5.

The highest reactivity was seen for monoclonal antibody 951–D10–B3 (antigenic site #3) and 951–C4–G2 (antigenic site #2) with peptide #2 (83-100). Both monoclonal antibodies react with free peptide as well as conjugated peptide . Lower reactivity (but a significant level) was found for two other monoclonal antibodies, 1G11–D11 (antigenic site #2) and 611–G10–D3 (antigenic site #3). Very low titers were found for peptide #2 versus monoclonal antibodies in antigenic site #1.

Table 5

Reactivity of Monoclonal Antibodies with Synthetic Peptides

ELISA Titers X10^{-3}

Antibody	Peptide #1	Peptide #2	Peptide #2 -KLH conjug	Peptide #3	Peptide #3 -OVAL conjug
Antigenic Site #1					
2BB10-G9	< 30	< 30	84	< 30	65
1G8-A11	< 30	< 30	48	< 30	80
3G12-C12	< 30	< 30	87	< 30	70
9E8-B2	< 30	< 30	80	< 30	87
821-B8-A8	< 30	45	115	< 30	87
2E5-C1	< 30	< 30	218	< 30	132
961-G8-H3	58	75	75	< 30	66
963-D3-G10	< 30	< 30	80	< 30	78
6B9-B8	< 30	38	76	< 30	66
Antigenic Site #2					
1G11-D11	< 30	56	529	< 30	193
951-C4-G2	< 30	1576	1012	< 30	43
823-D8-B11	< 30	< 30	< 30	< 30	< 30
M21C6	< 30	< 30	137	< 30	79
289/4-D5	< 30	< 30	121	< 30	60
Antigenic Site #3					
611-G10-D3	59	60	534	< 30	171
951-D10-B3	< 30	3416	2163	< 30	80
Unclassified					
611-B12-D10	32	83	178	< 30	122
961/6-B10	< 30	39	71	< 30	74

These data strongly suggest that residues 83 –100 of M–protein comprise a portion of both antigenic sites #2 and #3. Competitive inhibition assay defined these two sites as distinct from each other; however, they may be closely situated. One explanation may be the antigenic site #2 is, in part, comprised of a terminal portion of peptide #2 with antigenic site #3 comprised, in part, of the other terminal residues. Synthesis of overlapping peptides to the right and left of peptide #2 would aid in determining the exact epitopes recognized by monoclonal antibodies in these two antigenic sites.

The location of antigenic sites #1 has been localized to one of two regions based on A/PR/8/34–specific recognition of M–protein by a subset of monoclonal antibodies grouped in antigenic site #1 which includes the monoclonal antibody, 255–C1. Unlike the broadly reactive 2BB10–G9 (also in antigenic site #1) which had high titer against M–protein from every viral strain tested, 2E5–C1 was highly specific for A/PR/8/34, having a titer of 1:784,000 for A/PR/8/34 and a titer of less than 1:300 for all the other strains tested (except for A/USSR/90/77).

Analysis of sequence information of M–protein from A/Mallard/78, A/FPV/34, A/PR/8/34, A/Udorn/72, A/Bangkok/79, and A/WSN/33 shows that there are only three amino acid residues which are specific for A/PR/8/34.[6] These include residues #15, #116 and #231. Residues #15 is located in a hydrophobic stretch of amino acids and appears to be a highly unlikely candidate for the center of an epitope. Residues #116 and #231, however, both lie in the center of hydrophylic sequences and are good candidtates for antigenic site #1. Neither of these residues were included in the first set of three peptides prepared by Dr. Judd. Dr. Judd is analyzing these sequences with her computer program and will synthesize peptide segments containing these residues. ELISA analyses will be performed to detrmine reactivity of the peptides with monoclonal antibodies to establish the location of antigenic site #1.

II. Development of an ELISA System for Influenza Virus Detection Utilizing Monoclonal Antibodies to M–protein as the Capturing Agent

A number of parameters were explored in the development of a virus detection system for influenza utilizing monoclonal antibodies to M–protein as the capture antibody phase. Since M–protein is an internal component of the virion, conditions must be developed to optimize exposure of this antigen in the specimen while permitting maximal capture by the antibody phase. The type of plates must be examined to determine optimal adsorption with maximal retention of antibody activity. The use of ascites fluids directly as a source of antibody or the use of purified antibody must be explored. The optimal technique for purification of monoclonal antibodies must be developed. Conditions of buffer, time and pH of adsorption of antibody must be optimized. Various conditions of the assay at individual steps must be evaluated including (a) postcoating, (b) specimen pretreatment and exposure to antibody coated plates, (c) washing steps (d) reaction with sandwich antibody, and reaction with conjugate. All test development was performed using 'gargle' specimens from 'well' volunteers seeded with either egg or tissue culture (MDCK cells) propagated virus.

The overall design of the ELISA system for test development included purified monoclonal antibodies coated on microtiter plates as capture antibody, and purified antibodies from rabbit hyperimmune antisera to M–protein as the 'sandwich' antibody. The use of antibodies from two separate species allowed the use of the indirect system of

ELISA analysis; goat anti–rabbit antibodies conjugated to alkaline phosphatase were used as the indicator. This method eliminated the necessity for individually conjugating all monoclonal antibodies with alkaline phosphatase.

A. Variation of Parameters in Developing a Sensitive ELISA Test System

1. Coating the plates with 'capture' monoclonal antibodies

Purified monoclonal antibodies were adsorbed to Immunon 1 or Immulon 2 plates in the carbonate coating buffer, pH 9.0, or in phosphate buffered saline (pH 7.0) for 18 hours. Almost no adsorption occurred when PBS was used for Immulon 1 or Immulon 2 plates. Maximal capture on Immulon 1 plates was seen at relatively high coatings in carbonate buffer– 2 to 5 μg of purified antibodies in 100 μL (or 4 to 10 μg/well in 200 μL). Results with Immulon 2 plates were obscured by the propensity of M–protein to selectively adsorb.[7] Less M–protein (and virus) was detectable in the presence of antibodies than in their absence when Immulon 2 plates were used as the support. Under the condisitons of specimen treatment used in test development, M–protein did not selectively adsorb to Immulon 1 plates. In addition, the use of Immulon 2 plates resulted in much higher backgrounds due to their greatly enhanced 'stickiness'.

2. Postcoating of plates:

The standard technique for postcoating of plates has been to use 0.5% BSA in PBS in PBS–Tween buffer as the coating agent. We also examined the use of 0.5%–veal infusion broth (VIB) which we had found to result in lower background in earlier experiments with the selective adsorption technique. The VIB coating did result in lower background values but unfortunately also suppressed test sensitivity.

The length of the postcoating step was also examined. The standard 1 hour postcoating was compared with overnight postcoating using either BSA or VIB. The lengthier postcoating step resulted in higher sensitivity of the reaction. Possibly, renaturation of antibodies adsorbed to the plate may occur on longer exposure to the ideal conditions of high protein and neutral pH of the postcoating step. Adsorption of antibodies is performed under alkaline conditions and relatively low protein concentration – both nonideal conditions which favor denaturation of proteins.

3. Pretreatment of specimen: reaction with capture antibodies

A number of pretreatment regimens in addition to freeze–thawing were attempted including the addition of various levels of Triton X–100, reducing agents or a combination of both Triton X–100 and reducing agents. No enhancement of sensitivity was seen with the use of any of these modifications aside from addition of Triton X–100.

Detergent was required to expose to epitopes on the M–protein. The optimal level was determined to be 0.09% Triton X–100. This level of detergent permitted exposure of M–protein while retaining maximal Ag–Ab interaction.

Incubation of seeded specimens was performed at both 37°C and 56°C for half hour intervals. Elevation of temperature to 56°C resulted in decreased sensitivity of the reaction.

4. Reaction with 'sandwich' antibody

The 'sandwich' antibodies utilized were purified antibodies from hyperimmune rabbit antisera to M–protein. The use of purified antibodies rather than serum resulted in a considerable decrease in background reactivity of the test. In addition, as described earlier, the use of a second species permitted analysis by an indirect method allowing the use of a single conjugate for all tests with no necessity for direct conjugation of enzyme to the 'sandwich' antibody.

In addition, the antibody was adsorbed prior to use with a 'control' gargle specimen and allantoic fluid to eliminate reactivity with human or egg antigens which might be fortuitously present in the antibody population.

5. Reaction with alkaline–phosphatase conjugated goat anti–rabbit IgG antibody

Several anti–rabbit IgG and Fab alkaline phosphatase conjugates were obtained from Sigma, Boehringer–Mannheim, Hyclone Laboratories, and other sources. To date, we find the Boehriinger–Mannheim product to be superior. This product consists of affinity isolated goat antibody to rabbit IgG (H and L) with antibody–enzyme conjugation performed with gluteraldehyde. Goat serum was added to a concentration of 5.0 mg/mL by the manufacturer. The recommended dilution was *1:3000; we used the product at considerably lower dilutions of 500 to 1500.*

6. Use of biotin–streptavidin conjugates

The use of biotin–streptavidin systems (Bethesda Research Laboratory) was also investigated. Use of biotinylated secondary antibodies and the streptavidin conjugate (1) was not as sensitive as the use of alkaline phosphatase conjugates; (2) required an additional step (incubation with biotinylated antibodies before addition of the streptavidin conjugate); (3) needed a longer incubation period for the enzyme assay stage and (4) necessitated the use of a mechanical shaker in a walk–in incubator.

B. Additional Parameters:

1. The necessity for use of capture antibodies to detect M–protein

In our initial work on detection of influenza viruses through detection of M–protein using polystyrene beads we found that capture antibodies were unnecessary for adsorption of M–protein to the solid phase. Relatively harsh conditions of heat and detergent could be used to break open the virus, release M–protein and cause it to be adsorbed to the polystyrene beads.[7] In light of our earlier observations, we wanted to determine if the monoclonal antibodies were functioning as capture antibodies or was detection of M–protein occurring by selective adsorption of M–protein under the milder conditions employed in this assay.

Immulon 1 plates were left uncoated or coated with a combination of 2B–B10–C12 and 1G8–All purified monoclonal antibodies (2 μg of each antibody in 200 μl). A preparation of X–53a virus was assayed according to the usual protocol as described in Section IV Analysis of Clinical Specimens (this report, table 7). The results leave no question that the monoclonal antibodies are serving a capture role; the use of a coating of monoclonal antibodies greatly enhanced virus detection under the conditions used in this assay (see Figure 6).

2. Use of monoclonal vs. polyclonal antibodies as capture antibodies

We had observed earlier in test development that the use of monoclonal antibodies to M–protein as capture antibodies resulted in a more sensitive assay for virus than the use of purified polyclonal (rabbit) antibodies. Our system utilizes monoclonal antibodies to capture M–protein, rabbit antibodies to 'sandwich' the assay, and enzyme conjugated goat anti–rabbit antibodies for color detection. Since we had altered a number of parameters in test development, we decided to re–examine the use of polyclonal antibodies as capture antibodies and use the monoclonal antibodies as 'sandwich' with enzyme–conjugated goat anti–mouse antibodies for detection. The results were dramatically in favor of the continued use of monoclonal antibodies as the capture phase (see Figure 7). Almost no detection was achieved when polyclonal antibodies were used as the capture phase. In addition, the background was substantially higher when rabbit antibodies were used as capture antibodies.

3. Increase in level of Triton X–100

Triton X–100 could be increased to levels as high as 1.2% with an enhancement of approximately 40% in absorbance relative to the standard use of 0.09% at the stage of incubation of coated plates with the solution containing virus (see Figure 8). The additional Triton X–100 evidently helps to open up the virus and expose M–protein for captur by the antibody–coated wells of the Microtiter plate. The higher level could be used provided that the additional Triton X–100 above 0.09% was added after the sample was aliquoted into the well of the coated Microtiter plate. Direct addition of the higher level of Triton X–100 to the well before the sample resulted in lower readings perhaps due to solubilization of capture antibody.

C. Combination of Monoclonal Antibodies to Enhance Capture Assay System

A combination of 1G8–All and 2B–B10–C12 purified monoclonal antibodies were used as the capture antibodies on polystyrene plates for specimen analysis for the 1985–86 winter influenza season based on earlier results comparing various combinations available at that time. Additional hybridoma lines secreting antibodies to M–protein have been developed in our laboratory and we therefore undertook evaluation of our present collection. Various combinations of purified monoclonal antibodies were adsorbed to polystyrene microtiter plates (Immulon 1) with a total of 4μg monoclonal antibody coating each well in a 200 μl volume. Virus (X–53a, H1N1) in quantities of 0, 10, 25 and 50 ng was exposed to monoclonal antibody coated plates with mild distruption to expose M–protein and the captured M–protein sandwiched with purified rabbit polyclonal antibodies. Values are reported as a ratio relative to background, thus, 2.0 means an absorbance twofold above background, 6.0 an absorbance 6 fold above background, etc.

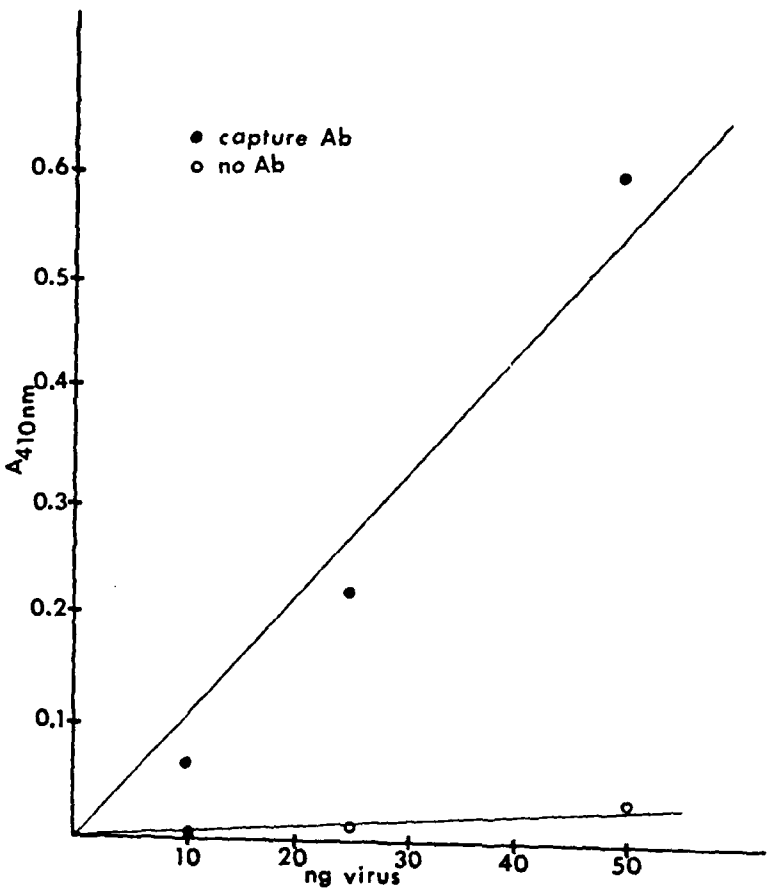


Figure 6. ELISA response to virus standard (X-53a) in the presence (●) or absence (o) of capture monoclonal antibody (2B-B10-C12 and 1G8-A11).

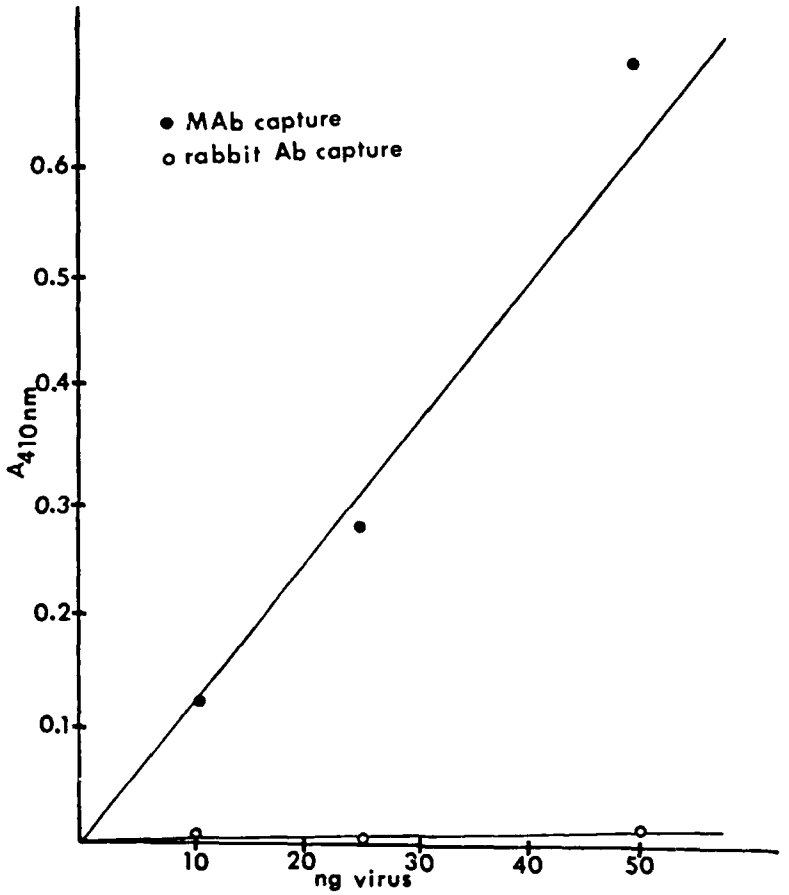


Figure 7. ELISA assay for standard virus (X-53a) in the presence of monoclonal antibody (●) or rabbit antibody (o) as the capturing antibody. Sandwich antibody is the opposite species; rabbit antibody in the first case (●) and monoclonal antibody (o) for the latter.

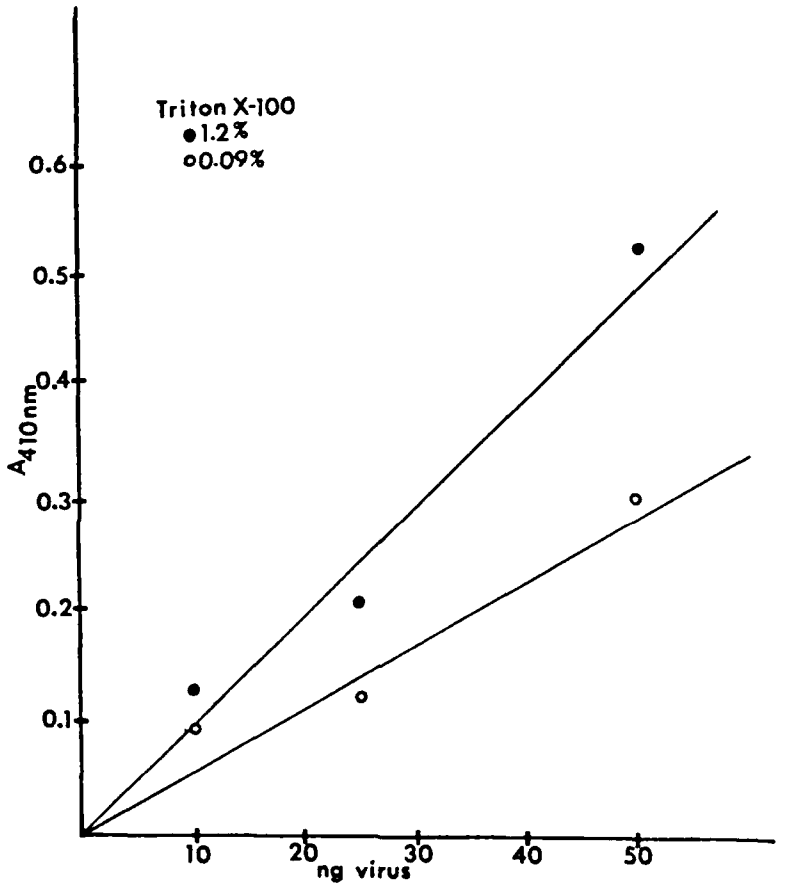


Figure 8. ELISA assay of X-53a virus standard in the presence of 1.2% Triton X-100 (●) or the lower level, 0.09% Triton X-100 (o).

Results of one set of experiments are shown in figures 9 and 10. Three monoclonal antibodies were coated on polystyrene plates, 611–B12–D10, 611–G10–D3 and 1G11–D11. Nine combinations were used including 2B–B10–C12 and 1G8–All. The optimal combination was 2B–B10–C12 and 611–G10–D3. 1G11–D11 and 2B–B10–C12 also performed well, however additional experiments with an H3N2 virus showed less sensitivity than the 2B–B10–C12 and 611–G10–D3 combination. Later experiments on epitope analysis supported the validity of both pairs of capture antibodies; 2B–B10–C12, 611–G10–D3 and 1G11–D11 evidently interact with different epitopes on M–protein.

III. Clinical Specimens

A. Isolation of Influenza Virus (H3N2) from Clinical Specimens (1984–85)

1. Mount Sinai Medical Center

Clinical specimens were obtained with the collaboration of the Pediatrics Department at Mount Sinai [Virus–Isolation Protocol in Appendix]. Collection of specimens began November 26, 1984, with the first isolate obtained December 10, 1984, from a child with pneumonia. Additional specimens were obtained over the Christmas–New Year holidays with the final specimen obtained January 16. Surveillance through April 30, 1985, failed to produce any additional influenza isolates in that season.

Our isolates preceded the peak activity in the U.S. by nearly a month; January was the peak reporting week for the nation.[8] Worldwide influenza activity was low during late 1984 and early 1985; Influenza A (H3N2) predominated in those outbreaks reported with infrequent outbreaks associated with influenza B; Influenza A (H1N1) isolates were rate.[9] The only reports of Influenza B activity thoutbreaks in March among school children in Hawaii.[10]

Ten positive specimens were obtained: a total of 81 specimens were collected during this period. All specimens were of the H3N2 subtype as typed and subtyped with the assistance of sera obtained from the CDC (developed to type A influenza viruses H3N2, and H1N1, as well as type B influenza virus).

Nasopharyngeal specimens were collected to permit analysis of samples with the highest possible virus titer. Specimens were quick–frozen and stored at $-70^{\circ}C$. Throat washings were also obtained from various laboratory personnel and student volunteers. All of these specimens were negative.

The use of MDCK cells permitted direct plaque isolation from clinical specimens. The procedure used for tissue culture was developed in Dr. E.D. Kilbourne's laboratory. Several isolates are shown in Figure 11. [MDCK Cell Protocol in Appendix]. Direct plaque isolates from the first four positive specimens obtained were submitted to the CDC for further analysis. All isolates were verified as H3N2 (A/Philippines/2/82–like) [CDC report in Appendix].[11]

Figure 9 and 10. Relative absorbance using different monoclonal antibodies (or combinations of monoclonal antibodies) as capture antibodies. A total of 4 µg monoclonal antibody was used to coat each well of the microtiter plate (Immulon I). The test virus was X-53a (H1N1) analyzed in quantities of 0,10,25 and 50 ng.

Figure 9

vs. X-53a (H1N1)

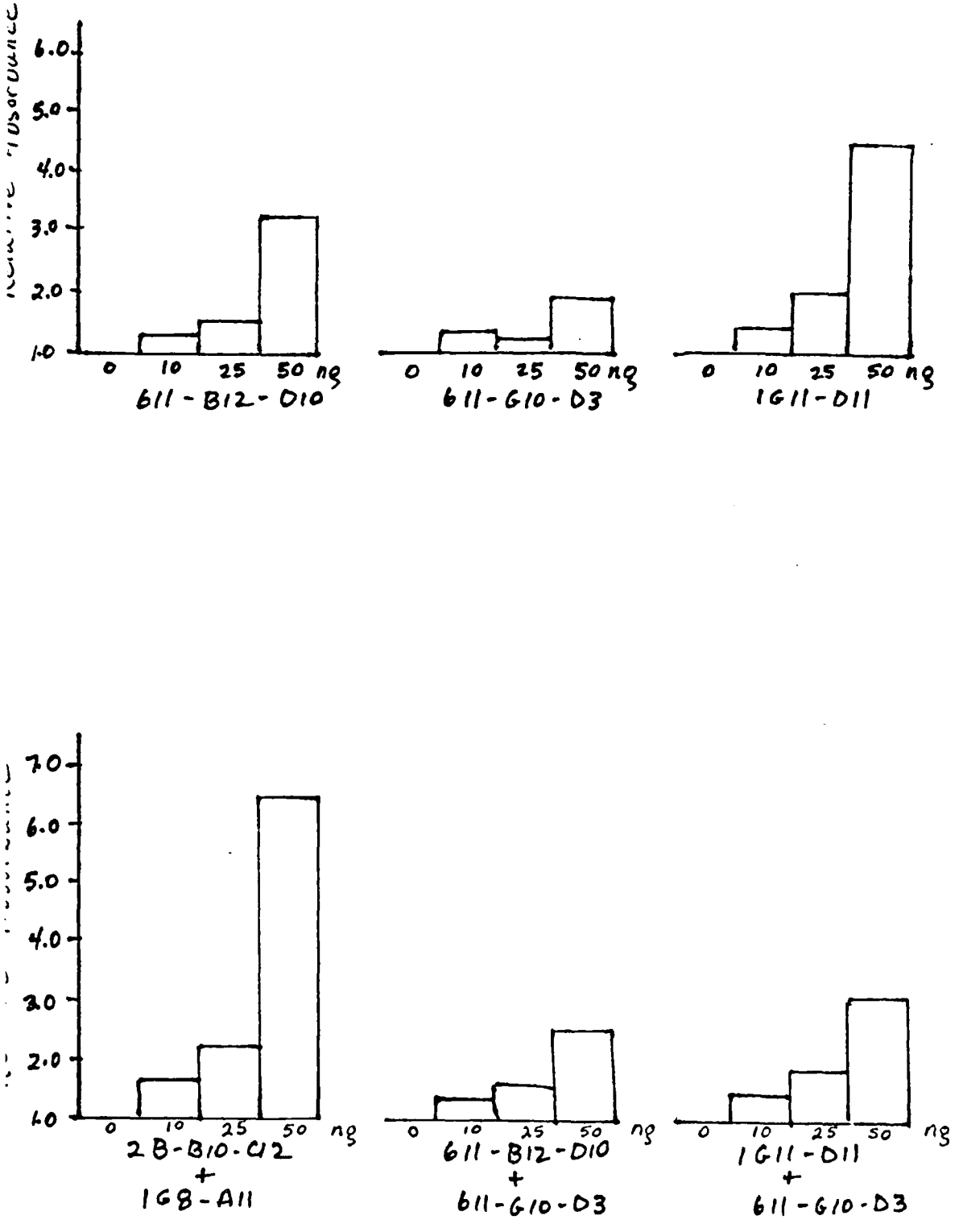
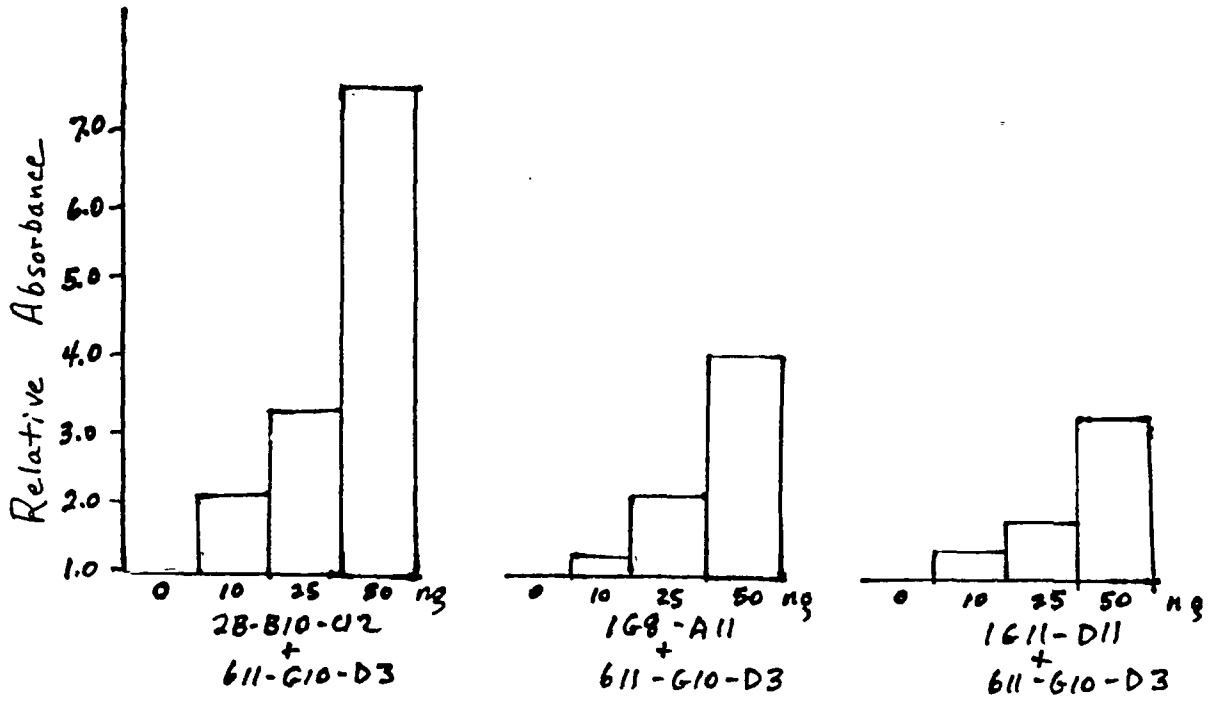
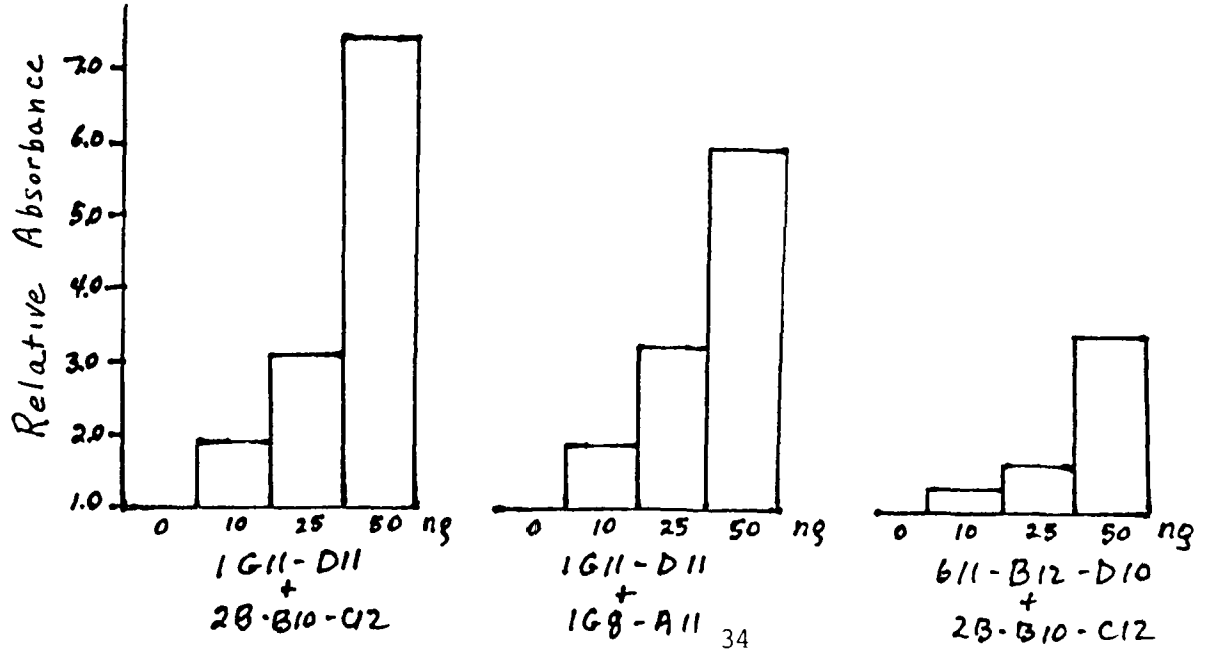


Figure 10

Vs. X-53a (H1N1)





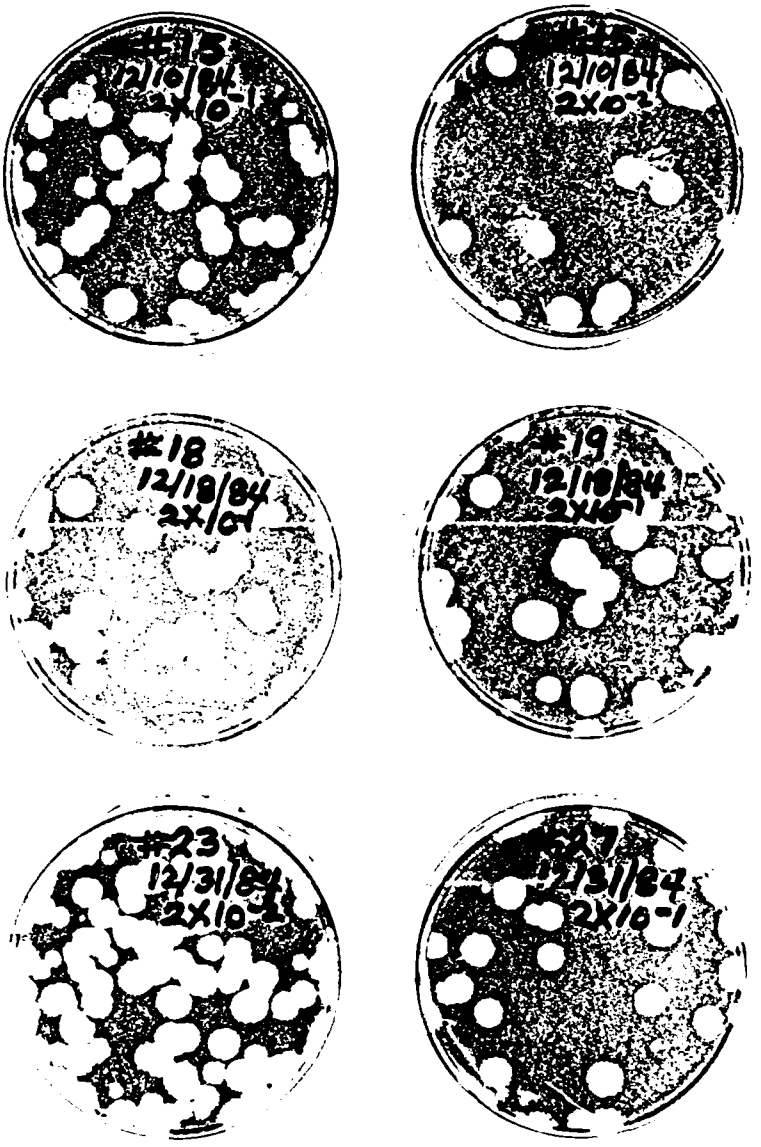


Figure 11. Direct plaque isolation of influenza virus (H3N2) from pediatric clinical specimens obtained at Mount Sinai. Specimens were diluted five- or fifty-fold and 0.2 ml applied to monolayers of MDCK cells. Cells were overlayed with an agar-medium mixture, incubated at 35°C for 48-72 hr. Plaques were visualized by crystal violet staining.

Plaque titers ranged from 750 to >5.0 X 10^4 PFUs/ml (the uppermost plaque number reached at our 1:50 dilution of a specimen). Attempts to retiter specimens after freezing and thawing were unsuccessful; plaque titer dropped one or more logs on freezing and thawing.

Nasal washes were performed with saline (see Appendix). Saline may not be optimal for maintaining the M–protein in solution for testing. 'Seeded' specimens have been stored at –70°C or –20°C for several months with various additives including serum albumin, gelatin, or veal infusion broth–gelatin. Based on ELISA results of stored 'seeded' specimens, the optimal storage medium will be selected for addition to specimens prior to freezing.

2. Specimens from Lowry AFB

Additional specimens were obtained from Dr. Meikeljohn's laboratory. This group has a well characterized collection obtained from their monitoring studies at Lowry AFB with both virus isolation and serology analyses performed on acute and convalescent sera. These specimens are throat washings performed with a veal infusion broth–gelatin mixture (2.5% V.I.B. and 0.5% gelatin in distilled water) and have been stored at –20°C.

B. Influenza Surveillance (1985–86)

In the Winter of 1985–86 we expanded our surveillance activities of individuals with respiratory tract infections considerably over the previous influenza season. In addition to our weekly sampling at Mount Sinai to obtain nasal washings from children, we enlisted the cooperation of the Director of Employee Health Services, Dr. Donald Smith, and obtained 14 throat wash specimens from Mount Sinai employees. We also obtained several positive specimens and performed the serotyping of isolates for Dr. Ed. Desmond of the Bronx VA Hospital. One set of five nasal wash specimens from Jersey Shore Medical Center was provided by a visiting fellow.

A total of 81 specimens were collected from the pediatric clinic which included 71 nasal wash specimens from children and 10 throat washings from adults. Three type A (H3N2) and two type B isolates were made from this population. One type A (H3N2) isolate was made from the 14 throat–wash specimens collected at Mount Sinai Employee Health services. Once type B isolate was made from the Jersey Shore Medical Center specimens. One type A (H3N2) and three type B's were serotyped from the Bronx VA isolates. Specimens from these various sources totaled 104; five type A (H3N2) and six type B isolates were identified.

We were also fortunate in gaining the cooperation of the Nassau County Medical Center Virology Laboratory, directed by Dr. Steve Lipson. We sampled possible flu specimens (throat and nasopharyngeal swabs). We tested a total of 185 specimens (throat and nasopharyngeal swabs) on a weekly basis from February 14 through March 14, 1986. These specimens were analyzed by ELISA and placed into tissue culture (MDCK cells 35 mmm dishes) to verify Dr. Lipson's results. pronounced CPE was associated with positive specimens (see figure 12). This group of specimens yielded 8 type A (H3N2) and 11 type B influenza virus isolates.

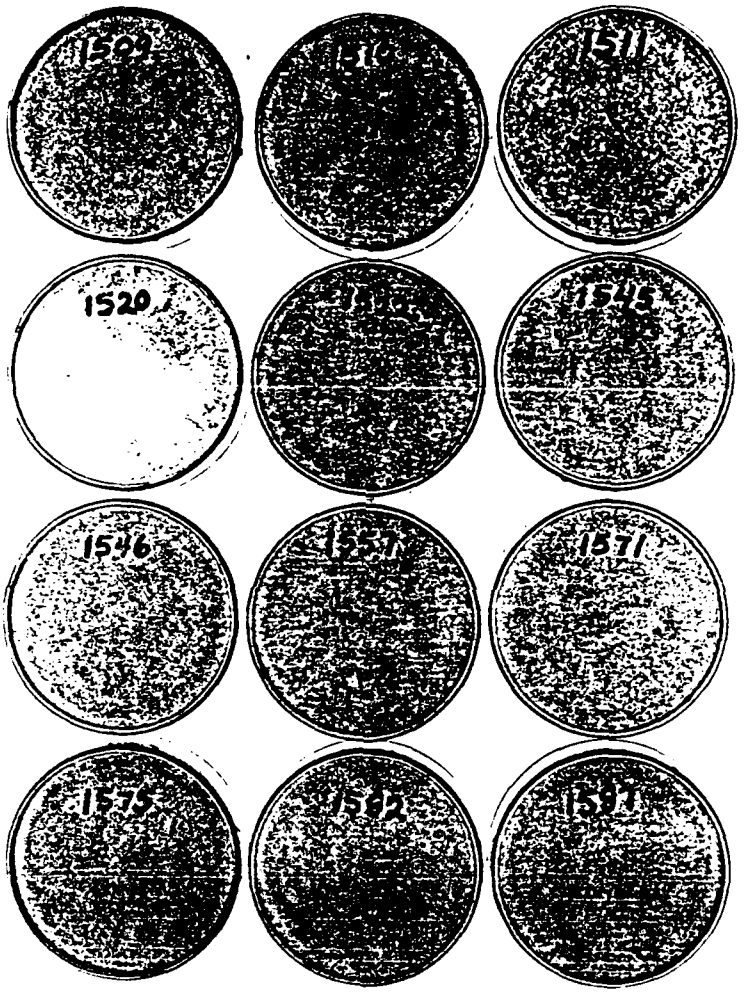


Figure 12. Tissue culture analysis of clinical specimens from Nassau County Medical Center. MDCK monolayers were exposed to 0.1 ml specimen (diluted 1:10), overlayed with media (no agar), incubated for three days and stained with crystal violet. Specimen 1520 was positive for type A influenza virus.

The specimens were collected by a variety of means. Specimens collected from children at Mount Sinai were nasal washes made with sterile saline. Adult specimens at Mount Sinai were saline 'gargles'. Specimens from the Bronx VA Hospital and Nassau County Medical center were throat and/or nasopharyngeal swabs placed in viral transport media. Nasal washings would be expected to have the highest antigen concentration, lower amounts of antigen would be found in throat washings and swabs.

The influenza season in the winter of 1985–86 began much later than in the previous year. The first isolate was made from a specimen collected January 21, 1986, and the last isolate was made from a specimen collected February 27, 1986. This is in contrast to the previous winter when the first isolate was made from a specimen obtained December 10, 1984, and the last isolate was made one month later from a specimen collected on January 10, 1985.

Early isolates in the season were type A (H3N2) influenza virus with later isolates predominantly type B. In the previous season only type A (H3N2) isolates were made.

A copy of our report to Dr. Maurice Harmon of the CDC and a summary of isolates made in our laboratory at Mount Sinai is presented on the following pages. Unfortunately, the member of isolates made at Mount Sinai was limited. Both type A isolates were found among the nasal wash specimens. The Nassau County specimens provided us with a much larger number of cases but the level of antigen present would also be expected to be much lower in the throat and nasopharyngeal swab specimens as compared with nasal wash specimens (Mt. Sinai).

C. Influenza Surveillance at Mount Sinai (1986–87)

A total of 83 specimens were collected from patients with respiratory infections at Mount Sinai from October 23, 1987, through January 13, 1987. The majority of specimens were nasal washes obtained from pediatric patients. However, only two influenza isolates were made from this collection of 83 specimens. One isolate was from a specimen collected on November 18, 1986, from a 14 year old female; the second isolate was from a specimen collected on December 2, 1986, obtained from a female pediatric resident. Both isolates were type A (H1N1 subtype) and closely related serologically to the A/Taiwan/1/86 viral strain.

The 1986–1987 influenza season appeared to be generally mild throughout the U.S. The percentage of deaths associated with pneumonia and influenza reached a peak of 6.2% which was below the peak reported for the previous two epidemic seasons of 7.2% and 6.6%, respectively.[12]

IV. Analysis of Clinical Specimens

A. Detection of Influenza Virus in Clinical Specimens (1985)

At present, we have several hybridoma lines secreting antibodies which are capable of capturing influenza virus in 'seeded' clinical specimens. These antibodies are capable of detecting virus to a sensitivity of about 10 ng/100 μl providing absorbance

THE MOUNT SINAI MEDICAL CENTER
ONE GUSTAVE L. LEVY PLACE • NEW YORK, N.Y. 10029

Mount Sinai School of Medicine • The Mount Sinai Hospital

Department of Microbiology

March 27, 1986

Dr. Maurice Harmon
Supervisor, Reference Lab
Division of Viral Diseases
Center for Infectious Diseases
Centers for Disease Control
Atlanta, GA 30333

Dear Maurice:

We've evidently seen the last of our flu cases for the season. For your records:

We monitored the pediatric clinic and in-patient population at Mt. Sinai on a weekly basis beginning Nov. 12 through Jan. 14 and collected a total of 37 specimens with no positives. From Jan. 21 through March 18 we collected an additional 44 specimens and found 5 positives. Type A (H3) isolates were made on 1/21/86, 2/4/86, and on 2/13/86. Type B isolates were made on 2/13/86 and 2/27/86.

Specimens were collected from employees attending the Mt. Sinai Health Service clinic from 2/6/86 through 2/11/86. Fourteen specimens were collected and one type A (H3) isolate was made on 2/11/86.

We also collected 5 specimens from children at the Jersey Shore Medical Center on 2/9/86 and obtained one type B isolate.

In addition we typed HA + isolates from specimens collected from adults at the Bronx VA by Dr. Ed Desmond. We typed a total of four isolates; one type A (H3) collected 1/29/86 and three type Bs collected on 1/29/86, 2/13/86 and 2/19/86.

The isolates have been amplified in MDCK cells. Please let me know if you would like any of them shipped to your lab.

Best regards,

Doris Bucher, Ph.D.
Associate Professor of Microbiology

3/27/86

TABLE 6

Specimen Summary

Mt. Sinai pediatric clinic and in-patient population

date	# specimens	#positive	
11/12/85	5	0	
11/19/85	3	0	
12/3/85	6(incl. 1 adult)	0	
12/10/85	3(incl. 1 adult)	0	
12/17/85	5	0	
12/26/85	5	0	
1/2/86	4	0	
1/9/86	3	0	
1/14/86	3	0	(37 specimens, 0 +)
1/21/86	6	1	type A (H3)
2/3/86	3(incl. 3 adults)	0	
2/4/86	7(incl. 2 adults)	1	type A (H3)-child
2/13/86	9(incl. 1 adult)	2	1 type A (H3)-child 1 type B-child
2/20/86	5(incl. 2 adults)	0	
2/25/86	3	0	
2/27/86	3	1	type B
3/6/86	5	0	
3/11/86	2	0	
3/18/86	1	0	(44 specimens, 5 +)
			total of 81 specimens

Mt. Sinai Employee Health Service (adults)

date	# specimens	# positive	
2/6/86	6	0	
2/7/86	3	0	
2/10/86	2	0	
2/11/86	3	1	type A (H3)

Jersey Shore Medical Center

date	# specimens	# positive	
2/9/86	5 (children)	1	type B

Bronx VA - HA+ specimens typed (all adults)

date collected	typing
1/29/86	1 type A (H3); 1 type B
2/13/86	1 type B
2/19/86	1 type B

values of two to threefold above background. The highest reactivity is seen with the hybridoma line 2B–B10–C12 (G9), both in terms of sensitivity and lack of background reactivity. 9E8–G10 also captures well but has a significant level of background reactivity.

Monoclonal antibodies from these hybridoma lines are reactive with X–53a (H1N1) from which the M–protein was derived and with an H3N2 isolate (15–2) from a clinical specimen obtained at Mount Sinai.

At this time, the test shows slightly higher values for positive specimens as compared with negative specimens from our Mount Sinai collection (see Figure 13). This low level of reactivity in specimens may be due to several factors. (1) The test is not sensitive enough to detect relatively low levels of virus present in a clinical specimen. The virus may be present at levels below 10 ng/100μl or 100 ng/mL which we can detect at present. The sensitivity of the test must be improved to detect these lower levels through decrease in background and increased sensitivity of reagents. (2) The M–protein present in the specimen may have become inaccessible with storage. We are using frozen specimens to establish the validity of the test. In 1986, assays are performed on specimens as soon as they are received using 2B–B10–C12 coated plates. The process of freezing and thawing or long–term storage of the specimen may have made M–protein inaccessible. We find a loss of 2/3 or greater in ELISA reactivity for seeded specimens stored at –70°. We have also found a dramatic decrease in sensitivity for our tissue culture propagated virus standards after storage over several months at –70°C. The M–protein may be adsorbing to the walls of the container. Stabilizing protein was not added to the specimens in 1984–85 but will be added in 1985–1986, depending on the results of our 'seeded' specimens stored with various protein additives. (3) The epitope(s) of M–protein recognized by the selected monoclonal antibodies may not be the epitopes displayed by M–protein in the human host.

B. Analysis of Clinical Specimens (1986)

Clinical specimens from Mount Sinai, Nassau County Medical Center and from Dr. Meikeljohn's laboratory at the University of Colorado at Denver were analyzed by ELISA assay. These clinical specimens represented three different collection techniques in various media; nasal wash in saline at Mount Sinai, throat and/or nasopharyngeal swab in viral transport media at Nassau County and gargle in veal infusion broth–gelatin at Denver. In all cases type A influenza virus containing specimens had a higher reactivity than those specimens from which no isolate was made. Furthermore, it is highly likely that influenza virus antigen may be present in a number of the negative specimens, even though no isolate could be made; thus, the negative specimens may have a higher mean reactivity than if all specimens were negative for antigen as well as viable virus.

1. Mount Sinai Specimens

Clinical specimens were assayed as collected during the influenza season according to the optimized ELISA protocol (Table 7) with plates coated with 2B–B10–C12 and 1G8–All. Unfortunately, both types A (H3N2) and B influenza viruses circulated with an overall lower rate of isolation than in the previous season. As a result, only three type A influenza virus isolates were made from the 81 nasal wash specimens collected from children at Mount Sinai.

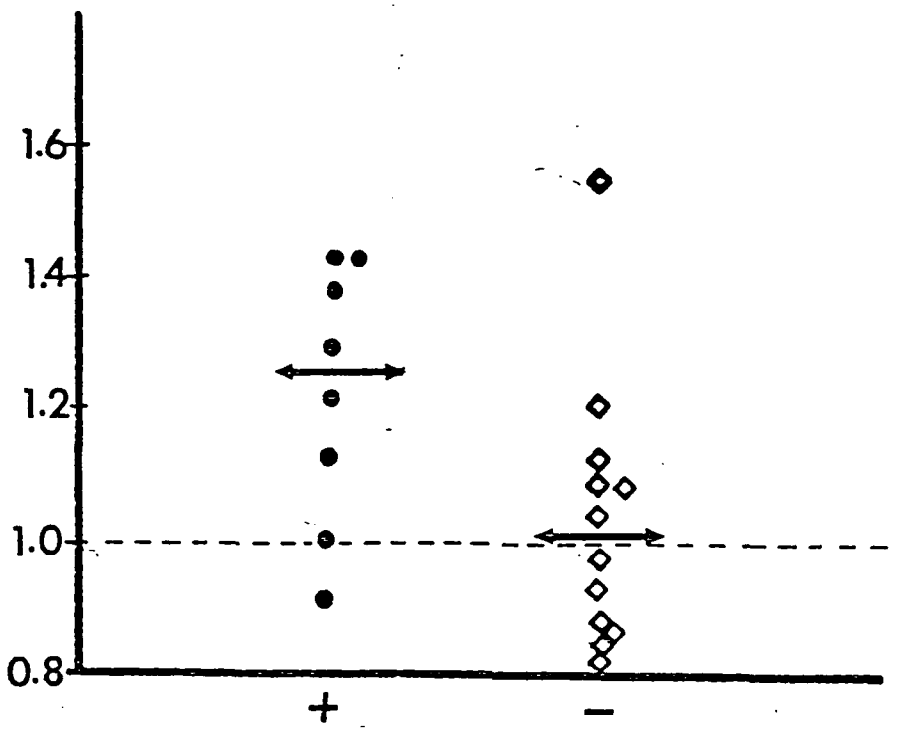


Figure 13. ELISA reactivity of clinical specimens, positive (+) and negative (-). Immulon 1 plates were coated overnight with 4 microg. 2B-B10-C12 purified monoclonal antibody and the Optimized rotocol followed (See Appendix). Absorbance values at the highest concentration of specimen were normalized by dividing by the background absorbance value. The median normalized value was 1.25 for positive specimens and 1.02 for negative specimens.

TABLE 7

ELISA Analysis of Influenza containing Specimens
Optimized Protocol (11/85)

1. Coat plates with 200 µl carbonate buffer containing purified IgG (2 to 5 µg/100 µl).

2. Wash 3 times with 200 µl PBS-Tween.

3. Postcoat with 200 µl of PBS-Tw containing 0.5% BSA. Incubate for one hour at room temperature.

4. Fill row 2 with 100 µl of PBS-Tw with additional 0.18% Triton X100. Fill remaining wells with 100 µl of PBS-Tw with additional 0.09% Triton X100.

5. Freeze-thaw specimen 3 times and warm in 37°C water bath.

6. Add 100 µl of specimen to row 2 and dilute in two-fold increments through row 12.

7. Incubate at 37°C for 1/2 hour.

8. Wash 3 times with 200 µl PBS-Tween.

9. Add 100 µl rabbit anti-M IgG at a concentration of 4 µg/ml in PBS-Tw and 0.5% BSA to all wells. The IgG was absorbed prior to use (see footnote[a]).

10. Incubate for one hour at room temperature.

11. Wash 3 times with 200 µl PBS-Tween.

12. Add 100 µl of goat anti-rabbit IgG conjugated with alkaline phosphatase at a dilution of 1:750 in PBS-Tween and 0.5% BSA. The conjugate was adsorbed prior to use (see footnote[b]).

13. Incubate for one hour at room temperature.

14. Wash 3 times with 200 µl PBS-Tween.

15. Add 100 µl p-nitrophenl phosphate substrate in diethanolamine buffer.

16. Incubate for one hour at room temperature in dark.

17. Stop reaction with 100 µl 1N NaOH and read absorbance with Titertek.

a. IgG is diluted to one ml with PBS-Tw and 0.5% BSA. One ml 'control' gargle specimen is added and held at 5-10° for 15 min. IgG is then diluted to final concentration of 40 µg/ml; 3% allantoic fluid added, and held an additional 15' at 5-10°. IgG preparation is clarified by centrifugation at 1500 rpm for 30 min.

b. Conjugate is diluted to 0.5 ml with PBS-Tw and 0.5% BSA and 0.5 ml 'control' gargle added. Conjugate is held at 5-10° for 15 min. and clarified by centrifugation at 1500 rpm for 20 min. The conjugate is then diluted to the final concentration.

The relative ELISA values (ratio of absorbance of specimen/absorbance of background) for the three specimens which were positive for type A influenza virus were 1.11, 1.10 and 1.06. These values were slightly above the mean of 1.00 for the 66 isolate–negative specimens assayed directly in ELISA.

2. Nassau County Medical Center Specimens

Nassau County Medical Center specimens were assayed according to the optimized protocol before freezing of specimens. The relative absorbance values were determined for all type A influenza containing specimens, type B specimens and a set of isolate–negative specimens collected on 3/14/86 at the end of the influenza season (see Figure 14). The median was slightly higher for the type A positive specimens than the type B or negative specimens with a median of 1.07 for type A, 1.04 for type B and 1.03 for the negative specimens. The means were 1.08 for type A, 1.03 for type B and 1.06 for negative specimens.

3. University of Colorado at Denver (Dr. Meikeljohn)

A set of type A (H1N1) positive specimens and negative specimens (negative by isolation and serology) obtained from Dr. Meikeljohn's laboratory were analyzed by ELISA assay. These specimens were collected during the winter of 1983–1984 through their surveillance of Lowry AFB. The specimens were obtained by having individuals with influenza–like symptoms gargle a veal infusion broth–gelatin mixture and collect the results.

Specimens were assayed according to the optimized protocol. (see Figure 15). The positive specimens had a median of 1.12 and a mean of 1.15; negative specimens had a median of 1.03 and a mean of 1.03.

C. Typing influenza virus clinical isolates through ELISA assay of M–protein

1. 2BB10–C12/1G8–All as capture antibody (1986)

The ELISA system for detecting influenza viruses through M–protein may have an important application for typing influenza virus isolates from clinical specimens. Eight specimens from which influenza virus isolates were made were apmplified in eggs and the allantoic fluids were analyzed by ELISA at four–fold dilution. These specimens wwere compared with eight specimens which were isolation negative for influenza virus (see Figure 16). All specimens were collected during the 1984–85 influenza season. All positive specimens did not react. No centrifugation or any other pretreatment was used before assay.

Virus isolates amplified in MDCK cells were also analyzed by the ELISA assay. Early isolates from the 1984–85 season showed high reactivity, later isolates from that season and from 1986 had very low reactivity (see Figure 17).

These results may indicate that the epitope on M–protein recognized by our monoclonal antibodies 2B–B10–C12 and 1G8–All may have mutated or that the gene for another M–protein may have been introduced into the circulating H3N2. Our standard H3N2 (15–2) isolated from a clinical specimen last year showed high cross–reactivity with M–protein from X–53a. If the epitope associated with the 1986 H3N2 has been altered, this would explain the relatively low degree of positivity seen in our direct assay of clinical specimens.

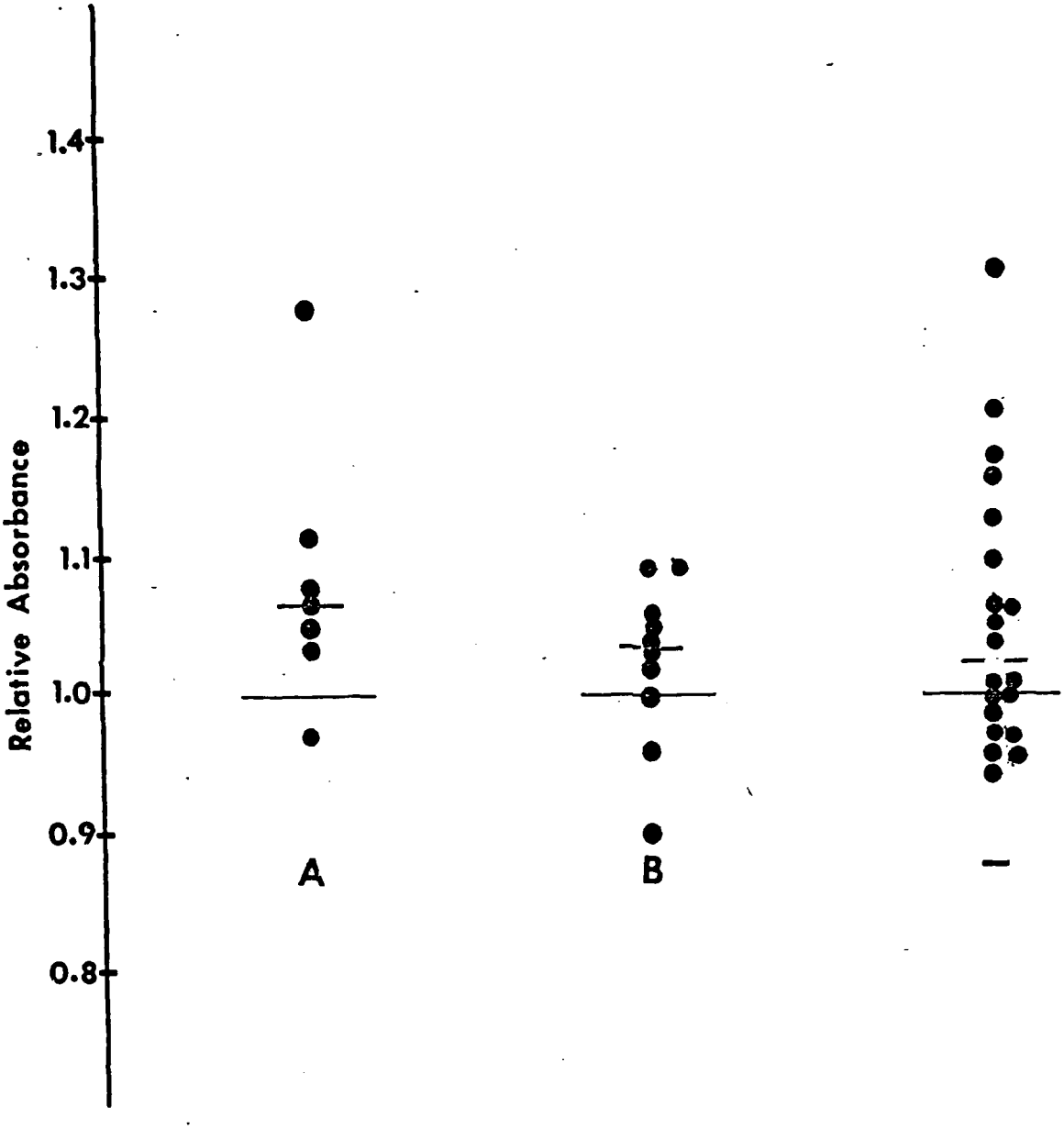


Figure 14. Specimens obtained from Dr. Steve Lipson of Nassau County Medical Center for the 1986 winter influenza season. Specimens were positive for type A, H3N2 (A), type B (B) or no isolate was made (-). Bars denote medians.

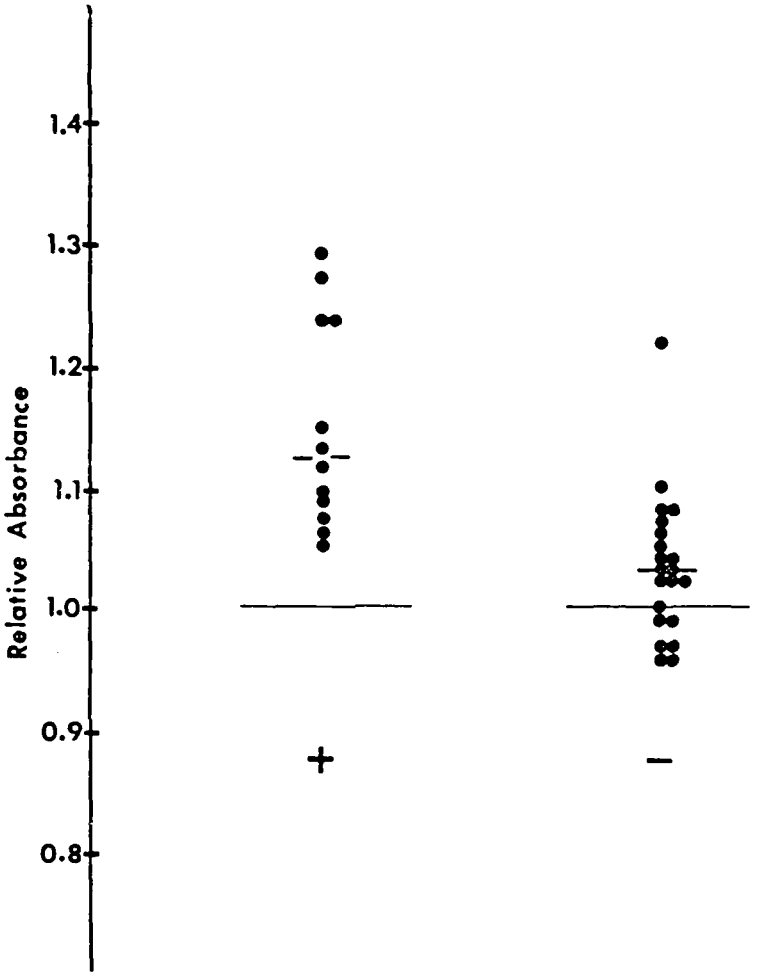


Figure 15. Specimens (H1N1) from Lowry AFB obtained from Dr. Meikeljohn at the University of Colorado at Denver from the 1983-84 winter influenza season. Specimens were analyzed according to the optimized protocol. Specimens were either positive for type A influenza, H1N1 (+) or negative both by isolation and serology (-). Bars denote medians.

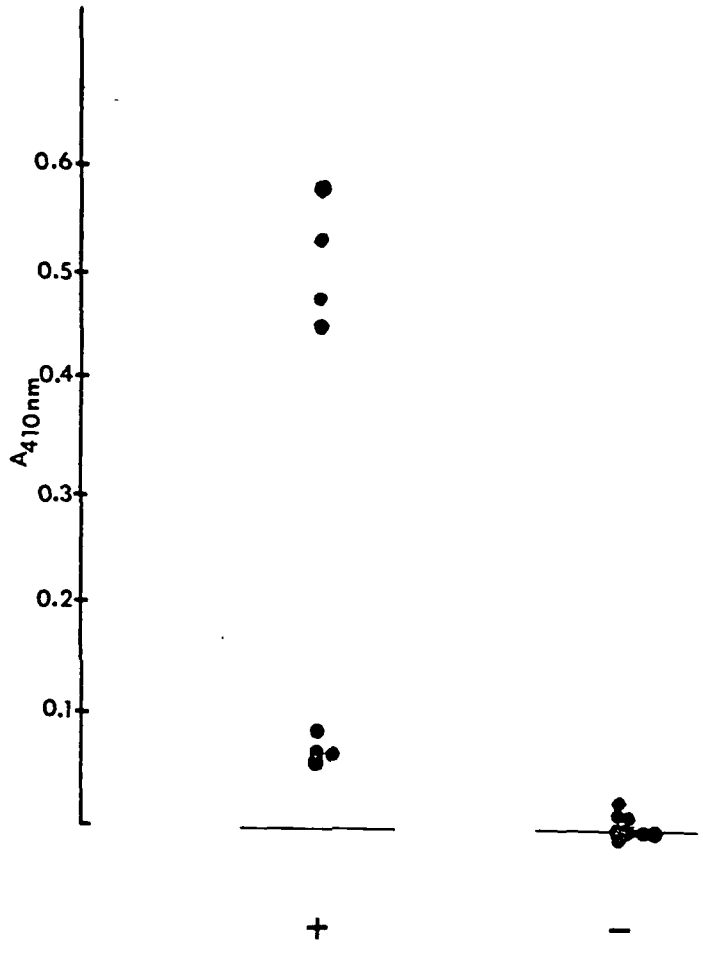


Figure 16. ELISA reactivity of specimens from the 1984-85 winter influenza season amplified in eggs and assayed at a 1:4 dilution. Specimens were either positive for type A influenza virus, H3N2 (+) or no isolate was made (-).

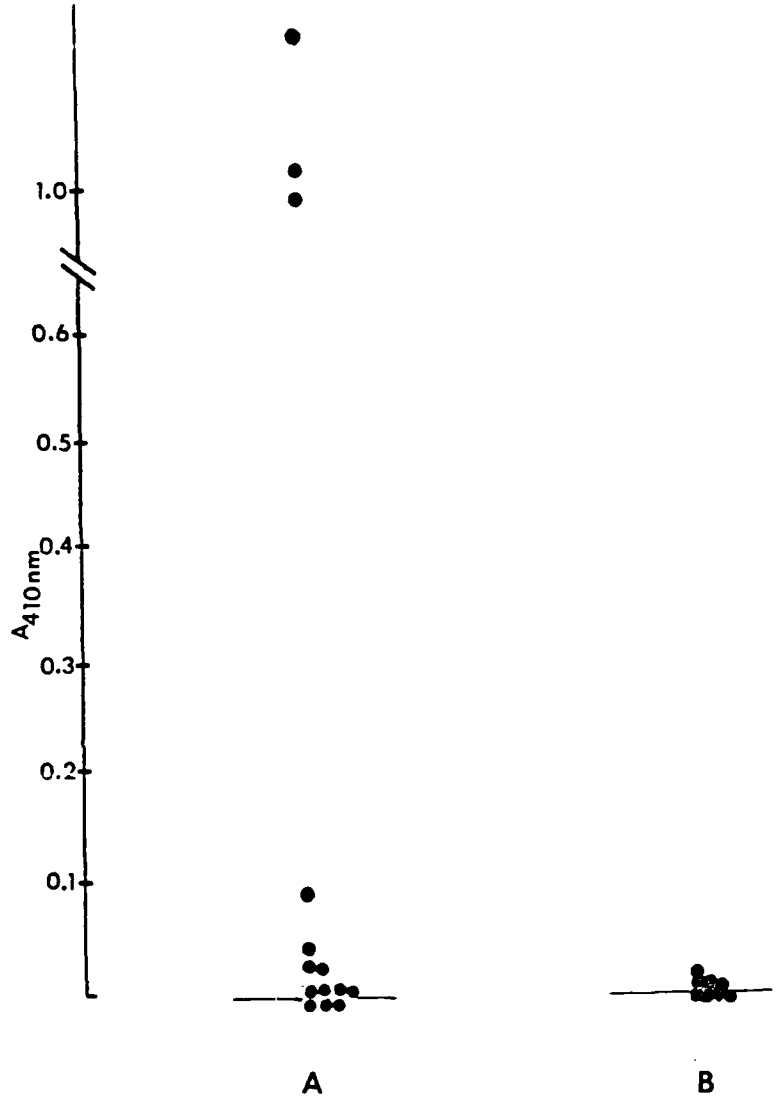


Figure 17. ELISA reactivity of specimens from 1984-85 and 1985-86 winter influenza seasons. Type A (H3N2) (A) and type B (B) influenza virus containing specimens were amplified in MDCK cells and then analyzed. Higher reactivity was seen among the early isolates from the 1984-85 season.

2. 1G11–D11 Monoclonal Antibodies as Capture Antibody (1987)

Based on the results of time–resolved fluoroimmunoassay (Section IV. D.) which suggested that 1G11–D11 performed well as capture antibody but was sensitive to Triton X–100, we re–examined the use of this monoclonal antibody for virus typing or tissue culture amplified specimens. No detergent was added to open the virus at time of exposure of specimen to capture antibody. Various test designs included the use of (1) 1G11–D11 as capture antibody, rabbit polyclonal antibodies as sandwich antibody with indirect detection by goat anti–rabbit alkaline phosphatase conjugated antibody; (2) 1G11–D11 and 2BB10–G9 combination as capture antibodies, rabbit polyclonal antibodies as sandwich antibody with indirect detection by goat anti–rabbit alkaline phosphatase conjugated antibody; and (3) 1G11–D11 as capture antibody with 2BB10–G9 directly conjugated with alkaline phosphatase as sandwich antibody.

An assortment of virus specimens amplified in MDCK or primary monkey kidney cells during isolation, were assayed by ELISA using the above test designs. The virus isolates included type A (H3N2 and H1N1) and type B influenza viruses. The optimal test design was (1), the use of 1G11–D11 as capture antibody with rabbit polyclonal antibodies as 'sandwich'; the results are shown in Figure 18. The high responder observed for type B is most likely a type A influenza virus isolate which was mislabelled.

D. Detection of Type A Influenza Virus in Clinical Specimens by Time–resolved Fluoroimmunoassay – Utilizing Monoclonal Antibodies to M–protein

Time–resolved fluoroimmunoassay (TR–FIA) is a potent tool in heightening sensitivity of viral detection systems. TR FIA utilizes a europium lable bound to an antibody with an EDTA derivative such as isothiocyanatophenyl–EDTA–europium.[13] Europium emits fluorescence with and exceptionally long fluorescence decay time (0.1–1ms).[13] After the immunometric assay has been completed the europium is dissociated from the antibody at low pH and measured by time–resolved fluorescence in a micellar solution containing Triton X–100, beta–diketone and a Lewis base.[13] The priniciples of solid–phase indirect TR FIA are the same as other indirect immunoassays utilizing capture antibody and sandwiched with a detector antibody.[14] Fluorescence is measured in a single photoncounting fluorometer equipped with a Xenon flash lamp; the commercially available instrument is a model 1230 Arcus fluorometer (LKB Wallach).[14] The lower limit of sensitivity of the assay was found to be 10 pg of type A influenza virus neucleoprotein when nucleoprotein specific monoclonal antibody was employed as capture and detecting antibody in TR FIA.[15]

Dr. Pekka Halonen of the University of Turku, Turku, Finland, performed preliminary testing of our panel of monoclonal antibodies to M–protein and found that a number of these served well as indicator antibodies in his system. Dr. Halonen invited me to visit their laboratory in March 1987, and work on application of our monoclonal antibodies to type A influenza virus detection in clinical specimens utilizing TR FIA.

A panel of 15 monoclonal antibodies reactive with the M–protein antigen of type A influenza virus were evaluated for their ability to detect purified M–protein by an ELISA technique utilizing peroxidase conjugated second antibody. Three monoclonal antibodies, 2BB10–G9, 1G8–A11 and 1G11–D11 were selected for use as detecting

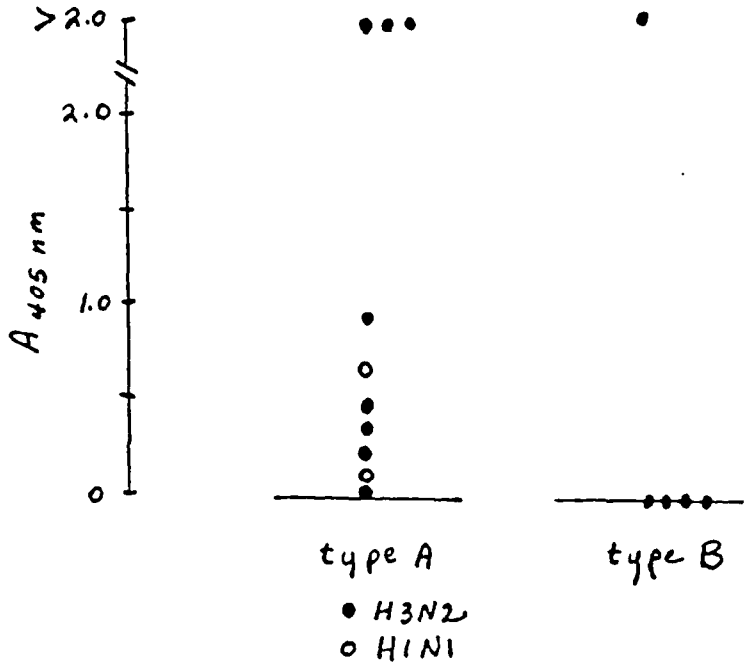


Figure 18. ELISA reactivity of specimens amplified in tissue culture by Mrs. Pat Graves of Dr. Gordon Meikeljohn's laboratory. Specimens were obtained as part of surveillance of Lowry AFB.

antibodies and conjugated with Europium chelate (LKB Wallach) for use in TR FIA. Seven monoclonal antibodies recognizing three antigenic sites of M–protein were utlized as capture antibodies. These included, site 1, 2BB10–G9, 1G8–A11, and 961–G8–H3; site 2, 1G11–D11 and 951–C4–G2 and site 3, 611–G10–D3 and 951–D10–B3.

Monoclonal antibodies were purified from ascites fluids by gel chromatography on an ACA–34 (LKB) column (2.5 X90 cm) in phosphate buffered saline containing 0.05% sodium azide. Protein concentrations ranges from 0.36–0.77 mg/ml. Monoclonal antibodies were dialyzed overnight versus phosphate buffered saline to remove sodium azide and concentrated to 6 to 13 mg/ml using the Centricon 30 (Amicon) unit prior to Europium labelling.

Monoclonal antibodies were conjugated with Europium chelate (LKB Wallach) as follows. Europium chelate (305nM) was added to one mg monoclonal antibody (6.1 nM) representing a 50 molar excess. Sodium carbonate (pH 10) was added in 1 ul aliquots to adjust the antibody–chelate solution to pH 9.2–9.5 and the preparation kept at room temperature overnight. The antibody chelate solution was chromatographed in 50 mM Tris, pH 7.75, 0.9% NaCl and 0.05% Na azide on a Trisacryl GF 2000 (LKB) column (1 cm X 50 cm) with continuous monitoring at A280nm. Antibody fractions were pooled and stabilizer DTPA–PUHD containing bovine serum albumin,, 9.2%, added. Label incorporated ranged from 5.7 to 9.2 Eu molecules/IgG.

Nasopharyngeal specimens had been collected during influenza epidemics in Finland in 1984–85 (H3N2) and 1986–87 (H1N1) and had been previously assayed by an ELISA technique. Isolates made during the 1984–85 outbreak included strains antigenically similar to A/Philippines/2/82, A/Coen/1/84, A/HongKong/3/84 and A/Finland/1/85. Isolates made during the 1986–87 period were similar to A/Singapore/6/86. Mucus specimen were diluted fivefold with phosphate buffered saline containing 20% fetal calf serum and 2% tween 20 and sonicated prior to ELISA analysis. Treated specimens were stored at $-20^{\circ}C$.

The assay was performed utilizing microstrips previously coated overnight at room temperature with 0.5–1.0 μg/well in 250 μul carbonate buffer (5mM Tris–HCl, pH 7.75, 0.09% NaCl and 0.05% Tween 20) utilizing the Nunc Immunowash 12 system. Plates were then postcoated with 0.1% gelatin in 50mM Tris–HCl, pH 7.75, and 0.09% NaCl. Plates were washed twice with TR FIA wash buffer. A volume of 50 μl diluent (50mM Tris–HCl buffer, pH 7.75, containing 0.9% NaCl, 0.5% gelatin, 0.01% Tween 40 and 20 μM diethylene triamine pentaacetic acid) was added to all wells followed by 50 μl of the specimen. Europium chelated monoclonal antibody (50 ng in 100 μl) was added immediately after the specimen and the microstrips incubated at $37^{\circ}C$ for one hour. The strips were washed six times with TR FIA wash buffer. Enhancement solution (LKB) was added to all wells followed by ten minutes shaking at room temperature to release the Europium. Fluorescence was assayed on an Arcus time resolved fluorometer (model #1230, LKB Wallach).

The optimal combination for detecting M–protein antigen proved to be 1G11–D11 as capture antibody and 2BB10–G9 EU as indicator antibody (Table 8). This combination also proved to be optimal for detecting virus directly in clinical specimens (Figure 19). Good detection was found with the combination 1G11–D11, 2BB10–G9 EU; 20 fold less sensitivity was seen for the combination of 2BB10–G9, 1G11–D11 EU (Table 9). One possibility is that Europium conjugation of 1G11–D11 damaged its ability to bind to M–protein (or perhaps

TRFIA Detection of Virus, M-protein or Nucleoprotein

Comparison of Use of Monoclonal Antibodies to M-protein or Nucleoprotein

TABLE 8

	Viral antigen detected	
	M-protein	Nucleoprotein
virus (100 ng)	91,000*	23,000**
M-protein (10 ng)	663,000*	------
Nucleoprotein (10 ng)	------	76,000**

* 1G11-D11, capture antibody; 2BB10-G9 Eu, detector antibody

** LKB anti-influenza A coated strips (antibody to nucleoprotein)

Table 5

Detection of M-protein by TRFIA: Comparison of Monoclonal Antibodies According to Antigenic Site Recognized

Capture Antibody	Detection of M-protein (10 ng) (counts) Detector Antibody (Europium Labelled) Antigenic Site 1 2BB10-G9	Antigenic Site 1 1G8-A11	Antigenic Site 2 1G11-D11
Antigenic Site 1			
2BB10-G9	13,902	10,132	16,319
1G8-A11	10,463	12,014	23,559
961-G8-H3	11,023	4,204	20,638
Antigenic Site 2			
1G11-D11	492,637	201,597	3,326
951-C4-G2	148,506	60,864	6,159
Antigenic Site 3			
611-G10-D3	99,390		3,341
951-D10-B3	204,159		5,552
no capture antibody	52,833	2,955	849
NP (10F-MAb)	30,858	6,503	1,884

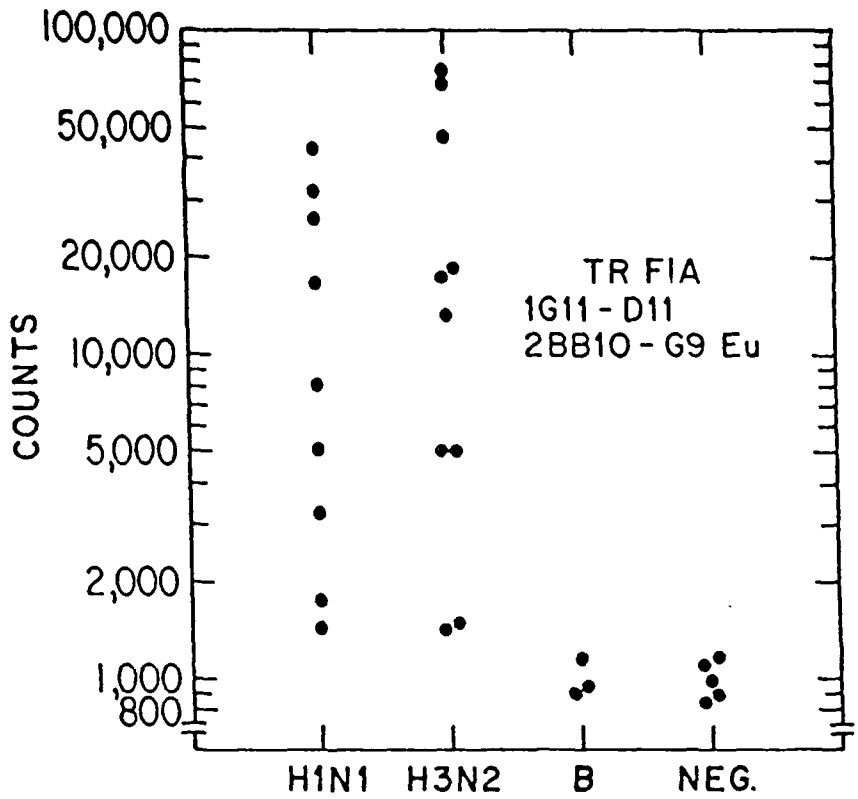


Figure 19

1G11-D11, capture antibody; 2BB10-G9 Eu, detector antibody
Protocol as described in text.

2BB10–G9 is not as avid at capturing M–protein as is 1G11–D11). The use of rabbit antibodies to M–protein antigen with indirect detection provided only very low (or no) sensitivity.

Addition of Triton X–100 enhanced detection of virus standards for some combinations of monoclonal antibodies but decreased sensitivity for the combination providing maximal signal in the specimen. Triton X–100 (or Tween 20) may have been denaturing 1G11–D11. Dilution of a nasopharynegeal specimen containing type A influenza virus and correction for dilution factor resulted in the curve of sensitivity shown in Figure 20; the low level of sensitivity found for the undiluted specimen suggests that the 2% Tween 20 previously added to the nasopharyngeal specimens was lowering sensitivity of the test, perhaps by denaturation of 1G11–D11 – capture antibodies.

The lower level of signal seen with M–protein antigen than for nucleoprotein may represent lower levels of M–protein antigen than nucleoprotein in the specimen (Table 10). Suboptimal processing of specimens for M–protein detection (i.e., addition of 2% Tween 20) and/or non–optimization of conditions for viral detection with the monoclonal antibodies to the M–protein antigen may also have been responsible for the lower level of sensitivity. Additional work should improve sensitivity of M–detection in TR FIA to levels equivalent to those found for NP. Detection through NP relies on a single monoclonal antibody; alteration in one amino acid may lower sensitivity or eliminate response entirely.

V. Immunofluorescence Analysis of M–protein in Influenza Virus Infected Cells

Immunofluorescence visualization of viral proteins can provide important information on the intracellular movement and localization of these proteins during the replicative cycle. this technique can also have an improtant practical application in the rappid detection of influenza virus through immunofluorescence analysis of cells obtained by nasal washing of individuals with respiratory infections. Immunofluorescence analysis has been utlilized as a method for rapid diagnosis of viral infections.[16] Investigators have reported special success in diagnosing respiratory syncytial virus infection.[17] Recently, McQuillin and associates reported the use of monoclonal antibodies in the rapid diagnosis of Influenza A and B viral infections.[18] Since M–protein is the most invariant of the antigens of type a influenza virus, monoclonal antibodies to M–protein can provide broadly reactive reagents for detection of all type A influenza viruses.

Two major observations were made with respect to M–protein localization within infected cells. (1) M–protein was found in the nucleus as well as the cytoplasm; previously there had been differing observations as to whether or not M–protein entered the nucleus during the replicative cycle. (2) M–protein associates with the actin filaments; monoclonal antibodies to M–protein stain the actin filaments in infected cells but not in uninfected cells.

We have developed a protocol for immunofluorescence analysis of infected cells on coverslips utilizing our monoclonal antibodies to M–protein (see Appendix for Immunofluorescence Analysis Protocol). Ascites fluids prepared to a number of our hybridoma lines have produced excellent visualization of infected cells with little or no background staining. Faint fluorescence is detected as early as 4 hours; maximal

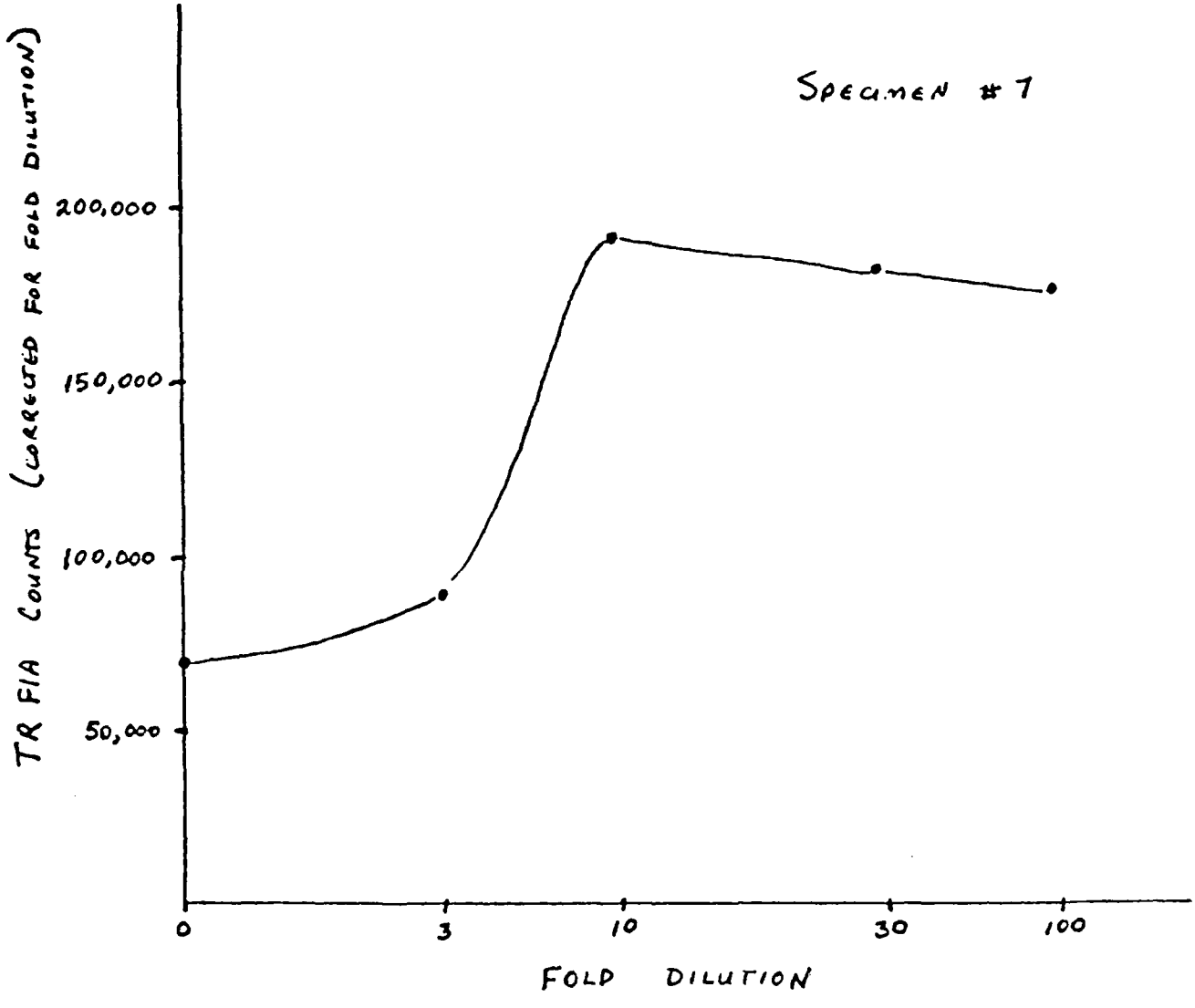


Figure 20. Effect of fold dilution of specimen (normalized to 0 dilution) on TRFIA counts obtained when 1G11-D11 is used as the capture antibody and 2BB10-G9 is the indicator antibody.

TABLE 10

Analysis of Clinical Specimens

Specimens		Counts (TR FIA)	
		M Ab	NP Ab
Positives*			
H1N1			
129935		3,145	17,016
130083		26,991	141,081
130084		16,475	52,408
130510		31,470	24,660
130512		8,140	4,081
130928		43,128	150,618
130994		1,425	9,036
131224		1,719	5,180
131250		5,077	1,784
	mean	15,286	45,056
	median	8,140	17,016
H3N2			
64117		17,569	11,368
64152		13,032	29,979
64205		1,406	11,233
64318		69,213	40,848
64368		5,016	14,604
64530		5,128	67,695
64656		45,643	70,587
64685		1,466	37,612
64687		18,117	22,630
64822		70,334	61,224
	mean	24,692	36,778
	median	15,300	33,796
Type B			
71319		1,116	643
71431		928	714
72559		940	664
Negatives			
131266		967	657
131268		1,172	644
131269		851	615
131925		1,133	841
132040		889	804

*as determined by ELISA analysis

intensity is reached at 6 to 7 hr. Ascites fluids which have been used successfully for immunofluorescence include those prepared for 2B–B10 (F1, C12 and G9), 1G11–D11, and 6B9–B8.

The procedure, briefly described, involves influenza virus infection of MDCK or CV–1 cells grown on coverslips at a multiplicity of infection 0.1 P.F.U/cell or greater. The CV–1 cells are larger than MDCK cells and permit easier localization of M–protein to specific regions of the cell. The use of fluorescein–conjugated goat anti–mouse IgG obtained from Cappel resulted in considerable enhancement of fluorescence staining. A fluorescence micrograph is shown in Figure 21 with CV–1 cells after 7 hr infection; ascites fluid prepared for the 1G11–D11 hybridoma line secreting antibodies to M–protein was used to detect M–protein. The fluorescence protocol employed in this series of experiments was modified from that reported in the Appendix of the 1985 Annual Report. Paraformaldeyde (3.75%) fixation was employed instead of fixation with acetone. This modification provided improved visualization of the cytoskeleton architecture.

Intense nuclear fluorescence of M–protein in X–53a virus infected cells is seen in both panels of figure 21. 1G11–D11 monoclonal antibody is used to visualize M–protein with fluorescein conjugated second antibody. 1G11–D11 recognizes antigenic site #2 of M–protein according to our competition assays. The X–53a virus is a reassortant influenza virus with the hemagglutinin and neuraminidase derived from A/NJ/76 (H1N1, swine flu) and the internal proteins including M–protein from A/PR/8/34.[2] The upper panel also shows considerable cytoplasmic fluorescence. The lower panel shows slightly more intense staining of the nucleoli as well as nuclear staining.

For comparison, nucleoprotein has been visualized by immunofluorescence with monoclonal antibody to nucleoprotein in figure 22. Only a small amount of rather granular staining is seen in the cytoplasmic region close to the nucleus.

The co–localization of actin filaments and M–protein was demonstrated in a series of double–labelling experiments utilizing rhodamine conjugated phalloidin and fluorescein conjugated second antibody with monoclonal antibody to M–protein (1G11–D11) as shown in figures 23 and 24. Phalloidin is a toxin which reacts specifically with actin filaments. In both figures 23 and 24 (lower panels), monoclonal antibody to M–protein interacts with the actin filaments (presumably through bound M–protein) in the cytoplasm as well as with M–protein in the nucleus. Some of the actin–containing microspikes surrounding the cytoplasmic membrane are also visualized with monoclonal antibody to M–protein.

Immunofluorescence co–localization of actin and M–protein is also seen in figure 25 (upper and lower panels). However,, in this case monoclonal antibody recognizing antigenic site #1 (2BB10–C12) is employed. The use of 2BB10–C12 also shows a slightly different pattern of staining with more intense diffuse staining of the cytoplasm, to the extent that the nuclei are obscured. This may suggest that the form of free M–protein in the cytoplasm exposes antigenic site #1 whereas antigenic site #2 is hidden and thus not available to react with 1G11–D11 monoclonal antibody.

The use of polyclonal (rabbit) antisera to purified M–protein for immunofluorescence localization results in the visualization of both actin filaments and nuclei (Figure 26). Intracytoplasmic fluorescence seems to be intermediate between that seen for 1G11–D11 and 2BB10–C12.

Figure 21. Immunofluorescence visualization of M-protein in influenza virus (X-53a) infected CV-1 cell monolayers at 5 hr post-infection. Visualization was performed with 1G11-D11 monoclonal antibody followed by the addition of fluorescein conjugated goat anti-mouse antibody (400 fold magnification). Same conditions for both upper and lower panels.

Figure 22. Immunofluorescence visualization of nucleoprotein in influenza virus (X-53a) infected CV-1 cell monolayers at 5 hr post infection. Visualization was performed with monoclonal antibody to nucleoprotein followed by the addition of fluorescein conjugated goat anti-mouse antibody (400 fold magnification).

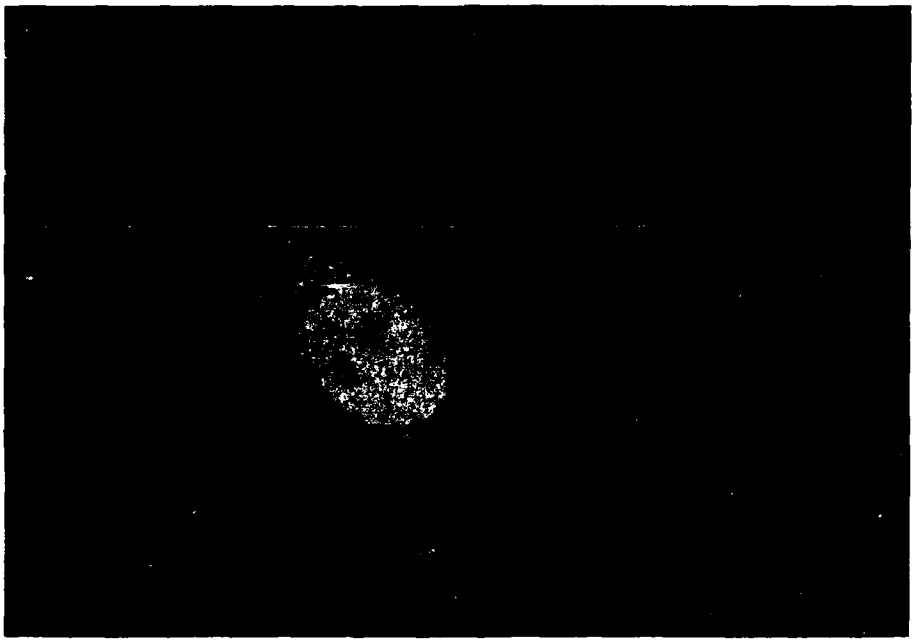


Figure 23. Immunofluorescence (double labelling) visualization of actin (upper panel) and M-protein (lower panel) in influenza virus (X-53a) infected CV-1 cell monolayers at 5 hr post infection (400 fold magnification).

Upper panel. Actin was visualized with rhodamine conjugated phalloidin

Lower panel. M-protein was visualized with 1G11-D11 monoclonal antibody followed by the addition of fluorescein conjugated goat anti-mouse antibody.

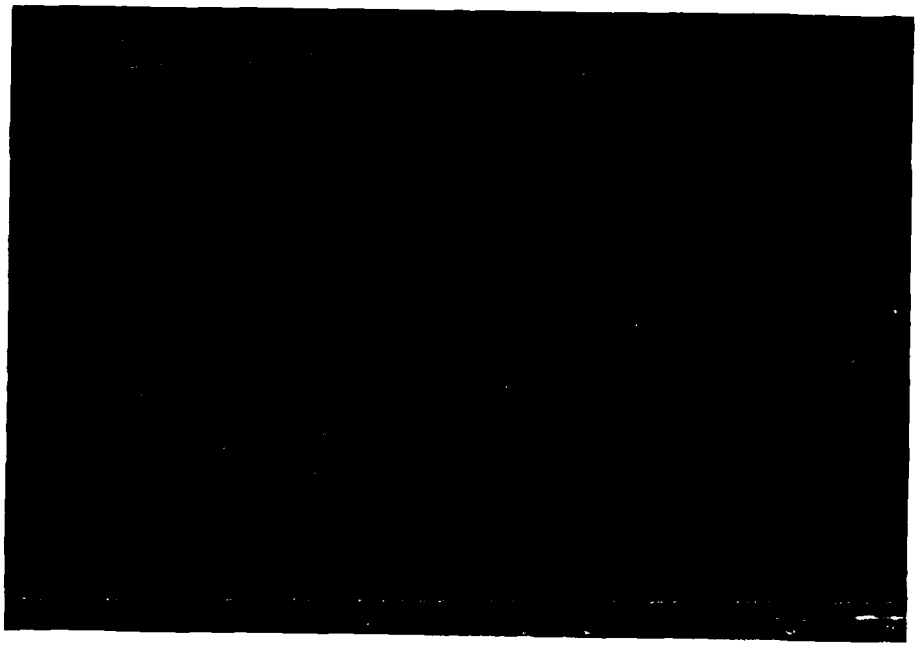


Figure 24. Immunofluorescence (double labelling) visualization of actin (upper panel) and M-protein (lower panel). Conditions same as for figure 3.

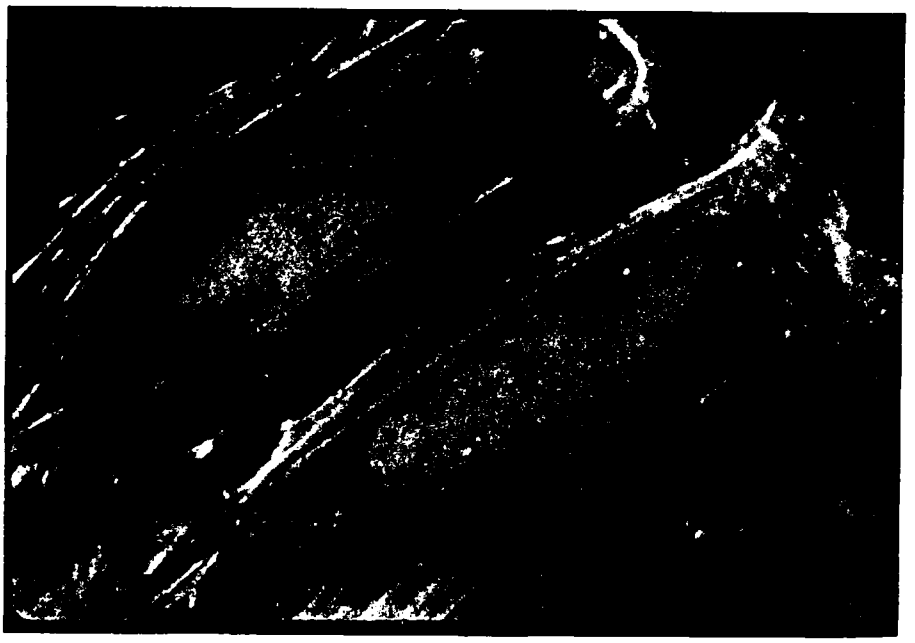


Figure 25. Immunofluorescence (double labelling) visualization of actin (upper panel) and M-protein (lower panel) in influenza virus infected cells. Conditions same as for figure 3 but 2BB10-C12 monoclonal antibody (antigenic site #1) used in place of 1G11-D11 monoclonal antibody (antigenic site #2).

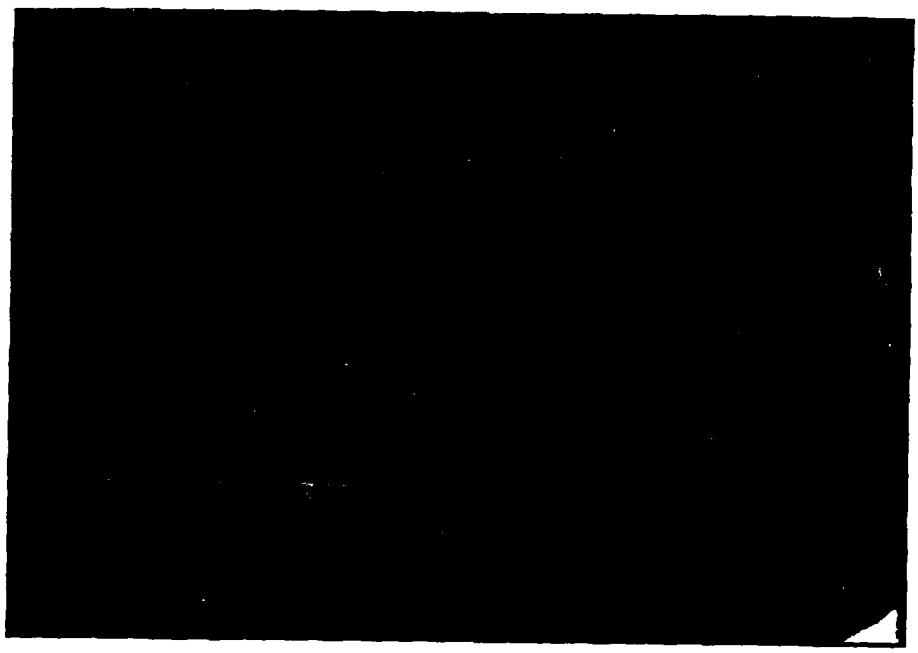
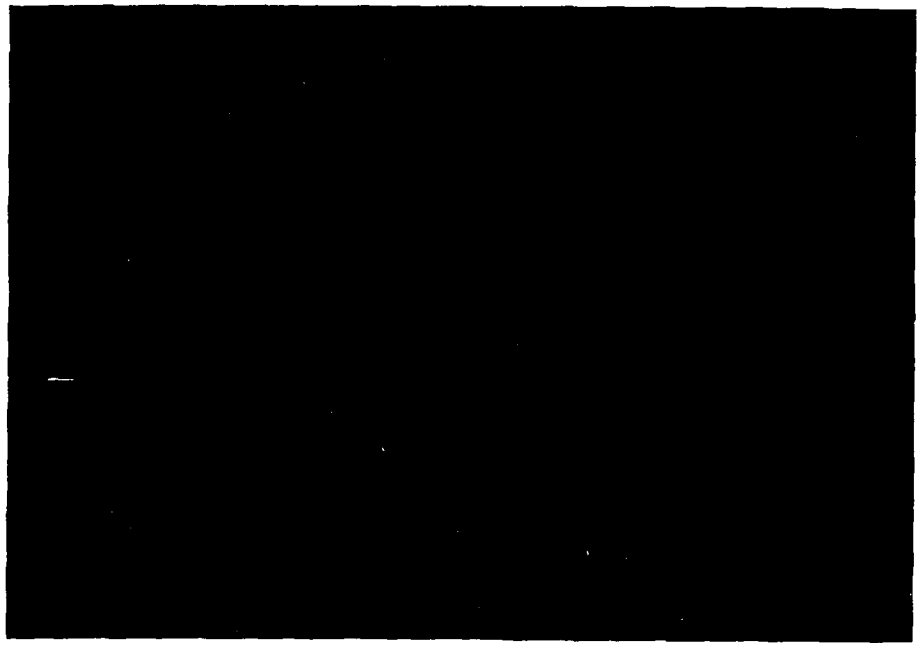


Figure 26. Immunofluorescence (double labelling) visualization of actin (upper panel) and M-protein (lower panel) in influenza virus infected cells. Conditions same as for figure 3 except rabbit (polyclonal) antisera to purified M-protein (#3948) was substituted for 1G11-D11 monoclonal antibody with fluorescein conjugated goat anti-rabbit antibody used for visualization of M-protein (lower panel).



Dr. Enzo Paoletti of the Wadsworth Center for Laboratories and Research of the New York State Department of Health in Albany, NY, generously provided us with his vaccinia recombinant containing the influenza virus M–protein gene (VP 273). The M–protein gene is derived from A/PR/8/34; the same source as the M–protein in our X–53a reassortant influenza virus. Several observations can be made concerning M–protein synthesized in association with vaccinia virus replication (as shown in figures 27 and 28).

(1) Synthesis of M–protein is much more rapid in CV–1 cells infected with the vaccinia recombinant than is seen for the X–53a virus reassortant. Intense fluorescence of M–protein is seen after 3 hr for the vaccinia recombinant as compared with 5 hr for the influenza virus reassortant, X–53a. This demonstrates the unusual strength of the promoter in the area in the vaccinia genome where the M–protein gene has been placed.

(2) M–protein enters the nucleus in the absence of other influenza virus proteins (figure 27, lower panel). None of the vaccinia proteins enter the nucleus (figure 7, upper panel); vaccinia is known to produce virus production 'factories' in the cytoplasm without involvement of the nucleus in viral replication. M–protein is also seen in association with the vaccinia 'factories' in the cytoplasm as shown by co–localization. One can conclude that the signal for transport of M–protein to the nucleus is contained within M–protein.

(3) No actin filaments are visualized except for those on the periphery of the cytoplasm (figure 28, upper and lower panels). It is known that vaccinia replication leads to the disaggregation of actin filaments.

Interaction of M–protein from a paramyxovirus with actin has been reported earlier by Tyrrel and coworkers.[19] This group reported that M–protein from Newcastle Disease virus would interact with purified actin. This group also reported on potentiation of anti–actin antibody production by NDV M–protein.[20] These investigators propose that a new antigenic site is exposed on the actin molecule by this interaction resulting in the induction of anti–actin antibodies in association with viral illness associated with influenza infection.

VI. Synthetic Peptides to M–protein

Synthetic peptides can be valuable tools in the development of rapid virus detection systems. They can be used as adsorbents ELISA detection systems to directly determine the epitope toward a monoclonal antibody is directed. They can serve in place of viral antigen as adsorbent in virus detection systems based on competitive inhibition. In such a system for viral detection, the peptide would compete with viral antigen in the clinical specimen for the limited amount of antibody added to the specimen. Peptides may also be valuable for use in epidemiologic screening of populations for antibodies to viral components as evidence of recent viral activity.

Dr. Armit Judd of S.R.I. International selected regions of the M–protein to be synthesized based on her analysis of hydropathicity data and secondary structure. (see Section I.D. Determination of Amino Acid Sequences of Antigenic Sites Recognized by Monoclonal Antibodies to M–protein, this report). These synthetic peptides represent the following segments of A/PR/8/34 M–protein: peptide #1, 66–78; peptide #2, 83–100, and peptide #3, 152–166.

Figure 27. Immunfluorescence (double labelling) visualization of vaccinia proteins (upper panel) and M-protein (lower panel) in CV-1 cell monolayers infected with the vaccinia recombinant (VP 273, containing A/PR/8/34 M-protein gene) at 3 hr post infection.

Upper panel. Visualization with rabbit (polyclonal) antisera to vaccinia virus followed by rhodamine conjugated goat anti-rabbit antibody.

Lower panel. Visualization with 1G11-D11 monoclonal antibodies to M-protein followed by fluorescein conjugated goat anti-mouse antibody.

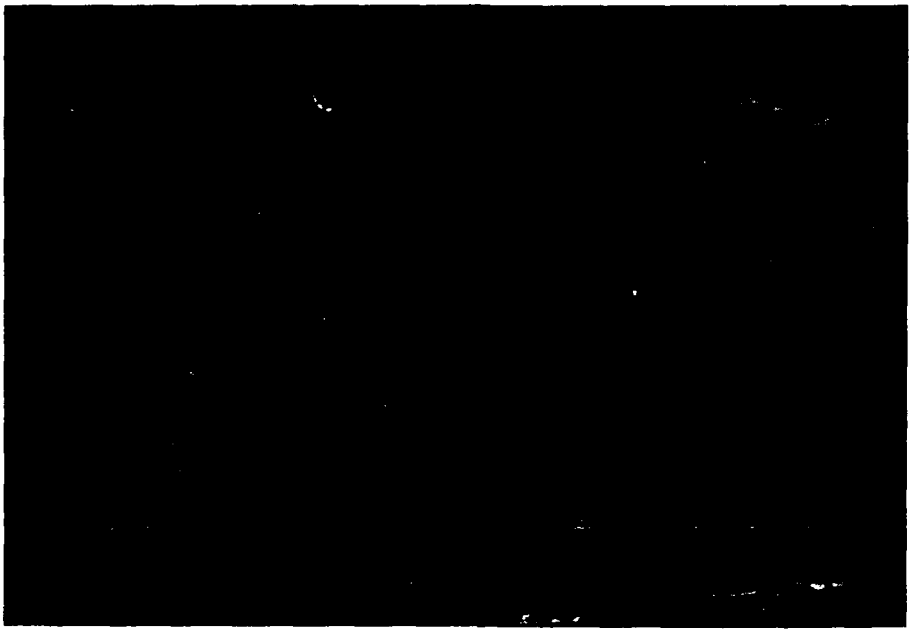
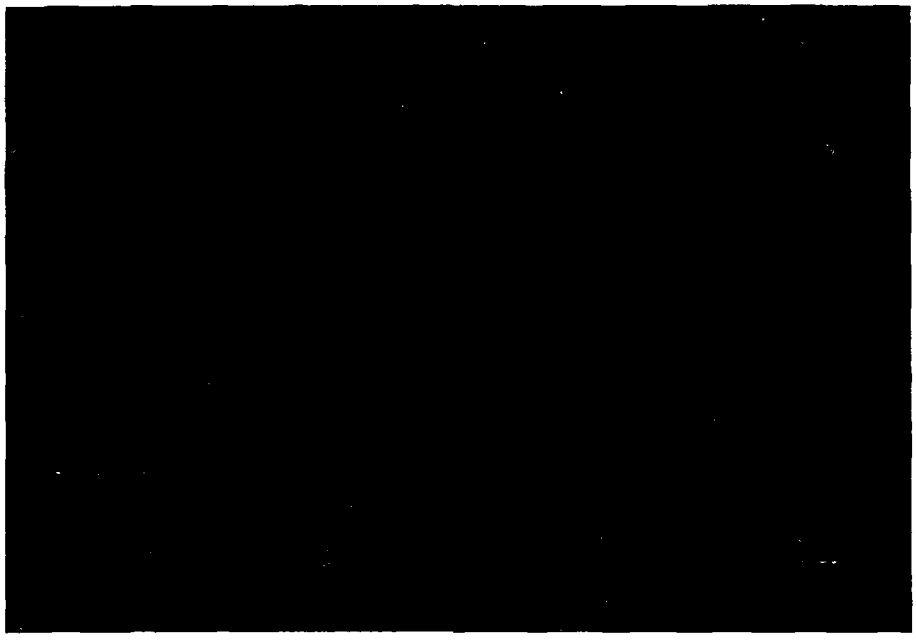
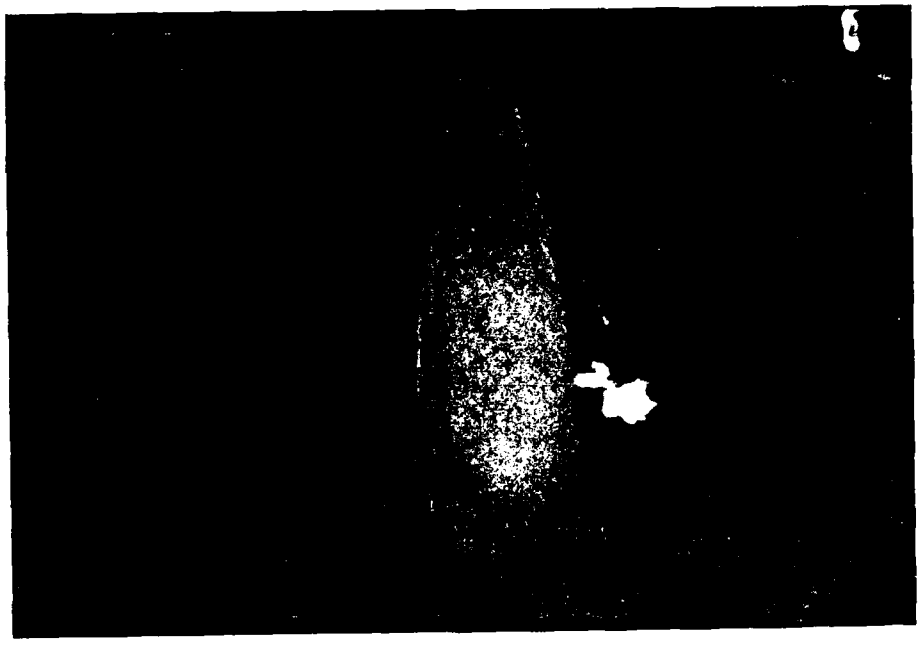


Figure 28. Immunofluorescence (double labelling) visualization of actin (upper panel) and M-protein (lower panel) in CV-1 cell monolayers infected with vaccinia recombinant expressing M-protein. Conditions same as for figure 7 except rhodamine conjugated phalloidin used for visualization of actin in upper panel.





The immunoreactivity of the peptides were evaluated by analyzing the ability of preformed antibodies in rabbit hyperimmune sera to bind to peptide coated Microtiter plates in an ELISA detection system. Immulon 2 plates (Dynatech) were coated with M–peptide (1μg/well) or M–protein (0.040 μg/well) in 0.1ml carbonate coating buffer for 18 hr. Endpoint determinations were calculated as absorbance values (A_{410nm}) which were three–fold above background absorbance. The data are reported in Table 11.

All three peptides were found to be immunorreactive as assessed by measurement of increased titers in ELISA assay. Peptide #1 had the greatest immunoreactivity of the three peptides with a 60.8 mean fold increase in titer of immune sera relative to preimmune sera, the mean increase for peptide #2 was 28.1 and peptide #3 was 12.9. The median fold increase was substantially lower for all three peptides with levels of 8.0, 4.2 and 4.3 found peptides #1, 2 and 3, respectively.

Synthetic peptide from M–protein, which represent distinct epitopes, may be useful in monitoring antibody response to influenza as described in the paper by Khan and co–workers (1982)[2]. Following infection with influenza virus, antibodies to M–protein rise more rapidly than those directed against the hemagglutinin. ELISA assay which use a 'cocktail' of synthetic M–peptides as coating on the solid phase could serve to monitor influenza activity in a community, nursing home or military base.

Synthetic peptides to M–protein may be valuable in the development of rapid virus detection systems for influenza virus based on competition type assays. In this type of assay, a fixed amount of antibody is added to a specimen. Subsequently, the mixture of antibody and specimen is then reacted with a known quantity of antigen on a solid support (i.e. Microtiter plate); the synthetic M–peptide could be used to coat the Microtiter plate as a synthetic M–protein antigen. In the competition type of assay, if virus (containing M–protein) is present in the specimen, the virus reacts with the antibody and decreases the ELISA titer on the microtiter plate coated with synthetic M–protein antigen.

Detection of viruses by competitive inhibition is limited by the availability of purified viral components. Liozner and co–workers (1983) demonstrated that M–protein could be used for competitive inhibition type detection for influenza virus infection.[21] This peptide could serve as an 'artificial' M–protein for development of diagnostic systems for influenza virus.

VII. Use of 'PR/8/Specific' M–protein Monoclonal Antibodies to Identify M–protein in Reassortant Viruses for Vaccine Production

Rapid adaptation of novel influenza virus variants (with potential for producing epidemic disease) to high growth capacity in eggs for viral vaccine production has become an important factor in cost–effective vaccines to protect against influenza, both in the civilian and military populations. Dr. Kilbourne and coworkers proposed this approach and his laboratory has been responsible for production of the high–yielding influenza virus reassortants used for vaccine production.[22] The methodology involves coinfection of the novel strain along with the A/PR/8/34 influenza virus strain to produce a reassortant virus with the surface antigens of the novel strain with one or more internal proteins donated by A/PR/8/34. The A/PR/8/34 strain grown to high

TABLE 11

Titers of Rabbit Antisera to M-Protein Following Immunization with Various Type A Influenza Viruses

Virus	M-Protein			Peptide #1			Peptide #2			Peptide #3		
	Serum Titers		Ratio	Serum Titers		Ratio	Serum Titers		Ratio	Serum Titers		Ratio
H1N1	Preimmune	Immune	Immune/ Preimmune	Preimmune	Immune	Immune/ Preimmune	Preimmune	Immune	Immune/ Preimmune	Preimmune	Immune	Immune/ Preimmune
A/NWS/33	178	161,889	909.5	177	39,136	221.1	188	6,387	34.0	605	7,205	11.9
A/NWS/33	104	336,634	3236.9	30	16,492	549.7	110	6,384	58.0	254	15,354	60.4
A/PR/8/34	810	313,096	386.5	718	62,486*	87.0	240	18,086	75.4	4583	19,583*	4.3
A/Swine/Cam/39	479	168,042	350.8	N.D.	1,041	-	657	2,177	3.3	N.D.	2,066	-
A/USSR/90/77	741	158,161	213.4	1278	4,658	3.6	2382	2,855	1.2	1954	5,449	2.8
A/Brazil/11/78	730	47,809	65.5	802	2,195	2.7	1746	5,223	3.0	741	1,516	2.0
H1N2												
X-7	143	50,957	356.3	41	627	15.3	671	1,951	2.9	257	254	1.0
X-7(F1)	226	68,592	303.5	242	1,308	5.4	1084	2,377	2.2	2197	2,004	0.9
PR8/HK #2071	188	15,405	81.9	206	537	2.6	237	805	3.4	1938	509	0.3
PR8/HK #2072	155	24,300	156.8	65	2,338	36.0	270	3,344	12.4	108	7,973	73.8
PR8/HK #3642	232	70,956	305.8	1013	7,223*	7.1	182	7,002	38.5	523	5,537*	10.6
PR8/HK #3643	153	23,183	151.5	N.D.	1,688	-	102	7,229	70.9	N.D.	1,210	-
PR8/HK #3644	73	35,700	489.0	36	1,759	48.9	30	6,766	225.5	176	2,239	12.7
H2N2												
A/RI/5-/57	142	20,889	147.1	117	2,161	18.5	217	774	3.6	609	2,321	3.8
H3N2												
A/Victoria/3/75	749	35,182	47.0	725	5,718	7.9	759	6,321	8.3	584	6,702	11.5
A/Texas/1/77	1010	2,046	2.0	712	225*	0.3	1298	685	0.5	639	176*	0.3
X-31	699	49,851	71.3	N.D.	772	-	357	1,707	4.8	N.D.	885	-
X-73	590	49,239	83.5	361	6,916	19.2	4880	2,116	0.4	471	5,974	12.7
X-79	2403	43,011	17.9	6,640	2,257	0.3	894	1,089	1.2	5750	3,031	0.5
H7N2												
X-74	207	18,528	89.5	82	672	8.2	53	684	12.9	136	1,361	10.0
Mean	501	84,674	373.3	779	9,218	60.8	818	4,198	28.1	1266	5,129	12.9
Median	229	48,524	154.2	242	2,257	8.0	314	2,616	4.2	605	3,031	4.3

* data not included in determining mean, median

yield in eggs, presumably from lengthy adaptation to the host from multiple egg passages since its isolation in 1934. The high growth capacity conferred on the reassortant virus has been associated with the acquisition of A/PR/8/34 M–protein.[23]

Our observation of 'PR/8" specific monoclonal antibodies in antigenic site 1 (see Table 4) suggested that these monoclonal antibodies could be helpful in identifying the source of M–protein in reassortant viruses prepared for vaccine purposes. Dr. Kilbourne and his technical assistant, Ms. Barbara Pokorny, had prepared a high yielding reassortant virus of the A/Leningrad strain (H3N2) for vaccine production. Microtiter plates (Immulon 1) were coated with 100 ng of test antigen; M–protein, A/PR/8/34, A/Leningrad/36086 or X–91 (the putative high yield reassortant of A/Leningrad/36086). Monoclonal antibodies selected from Table 1 with broad reactivity, 2BB10–G9 and 1G11–D11, and narrow reactivity ('PR/8 specific'), 2E5–C1 and 961–G8–H3 were titered against all four test antigens – (A/Leningrad/360/86 and X–91 were coded) and ELISA analysis performed.

The A/Leningrad/360/86 parent strain showed little or no reactivity with the 'PR/8 specific' monoclonal antibodies, whereas the X–91 reassortant virus had titer similar to those found with A/PR/8/34 (Table 12). Therefore, the X–91 reassortant virus derived its M–protein from the A/PR/8/34 parent, and presumably has achieved optimal growth potential.

Table 12

DETERMINATION OF M-PROTEIN DONOR
FOR A REASSORTANT VIRUS (X-91)

	Monoclonal Antibody			
	Broad reactivity		'PR/8 specific'	
Test Antigen	2BB10-G9	1G11-D11	2E5-C1	961-G8-H3
M-protein	1,428,000	3,836,000	829,000	1,779,000
A/PR/8/34	283,000	413,000	555,000	1,335,000
A/Leningrad/360/86 (H3N2)	180,000	295,000	< 300	2,000
X-91	829,000	1,250,000	908,000	624,000

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A P P E N D I X M A T E R I A L

Virus Isolation from Specimens (1)

1. Specimens are collected from children with symptoms of upper respiratory tract infection and fever who are attending the Pediatric Clinic or hospitalized in the Pediatric Ward. Five ml of sterile saline are instilled into the nasal passages and the washings collected with a mucous extractor (order as ear syringe from General Stores). Specimens are transported to the laboratory on ice in large scintillation vials (12ml).

2. One-half ml of penicillin and streptomycin (M.A. Bioproducts, 5000 units pen. + 5000 units strep per ml) are added to each specimen on return to the laboratory. Specimens are emulsified by repeated uptake and injection from one ml syringe.

3. Specimens are transferred to small scintillation vials (4-5ml) for storage.

4. Specimens are diluted 1:5 and 1:50 with PBS + Bovine Albumin for virus isolation. The original specimen is quick-frozen and stored at - 70°. (See MDCK protocol).

5. MDCK cells -
   Confluent MDCK cell monolayers in 60 mm dishes are infected with 0.2ml of the diluted specimen for 1/2 hr. @35°. Two dishes (one at each dilution) are used for each specimen. The dishes are then incubated at 35° for several days and observed for the appearance of plaques.

6. Embryonated eggs (2)

Specimens at the 1:5 dilution are also injected into the amnion and allantoic cavity of three - four 10 d. eggs. Specimens are injected with a one ml syringe equipped with a 25 gauge 1-1/2" needle, with 0.1 ml injected into the amnion and 0.1 ml injected into the allantoic cavity.

7. After 42 hr, following refrigeration of eggs, the allantoic fluids are harvested separately with a 3 ml syringe equipped with a 23 gauge needle. All fluids are harvested sterilely.

8. The hemagglutination test is performed on both amniotic and allantoic fluids with 0.5% human red cells in Microtiter plates. Positive fluids are quick-frozen and stored at -70°.

9. Infected MDCK plates are examined for any hemadsorption using two ml 0.2% human red blood cells (type 0). Red blood cells are allowed to adsorb for 15 min. Plates are washed two times with PBS. [Evidence of infection with para-influenza viruses can be obtained through the use of guinea pig or chicken red blood cells]. Plates are stained with crystal violet for examination of plaques.

MDCK Cells (Canine Kidney)

Subculturing - 2x weekly

1. Wash bottles with 10 ml 1X PBS
2. Add 1.5 ml EDTA/Trypsin
3. Incubate at 37° for 10-20 min.
4. Shake to dislodge cells and add 5 ml growth medium (MEM + 10% Δ fetal calf serum)
5. Pool in centrifuge tubes and centrifuge 2000 RPM 5 min
6. Remove supernatant. Resuspend pellet in 10 ml growth medium (depending on number of bottles). Pipet to break up clumps
7. To count - 0.1 ml cells + 0.9 ml PBS

   For flasks

   $2.2 - 2.5 \times 10^6$ cells + 20 ml growth medium

   For dishes

   $1.1 - 1.3 \times 10^6$ cells diluted in 5 ml medium for each dish

Plaques

1. Wash dishes with 2 ml 1X PBS
2. Inoculate dishes 0.2 ml virus diluted in 1X PBS/BA
3. 30 min adsorption 37°C
4. 4 ml agar overlay + 2 µg/ml trypsin
5. After agar has hardened place dishes with cell sheet up (invert dishes)

   [If antiserum is incorporated in overlay, dilute antiserum in 1X MEM

   9 ml agar overlay + 0.2 ml antiserum]
6. Plaques visible on day 3-4
7. Dishes may be stained
   a) remove agar
   b) stain 10 min - crystal violet (0.1% in 20% ethanol)

Crystal Violet Stain

Stock Solution -

1% crystal violet in 20% ethanol

1g crystal violet
20 ml 95% ETOH
75 ml H_2O

Working Solution - (0.1% crystal violet in 20% ethanol)

10 ml 1% crystal violet
90 ml 20% ethanol

MDCK MEDIA

Reagents

A. Growth Medium

MEM + Δ 10% FCS

10 X MEM (GIBCO)	50ml.
Dist. Water	375 ml.
5% Na HCO_3	15 ml.
100 X Pen & Strep. (MAB)	5 ml.
Δ FCS (fetal calf serum)	50 ml.
L-glut 100 x (MAB) 200 mM in saline	5 ml.
Hepes (1.0M) (MAB) in saline	5 ml.
	500 ml.

B. 2 X MEM + B.A. (for overlay)or to make 1X MEM

10 X MEM	100 ml.
35% bov. alb.	6 ml.
L-glut 100 X	10 ml.
5% Na HCO_3	30 ml.
Hepes (1.0M in saline)	10 ml.
Dist. Water	344 ml.
100 X P & S	10 ml.
	500 ml.

C. Trypsin - EDTA (Versene)

1% Trypsin in PBS (DIFCO TRYPSIN)	10.0 ml.	2.0 ml.
2% EDTA in DW	0.5 ml.	0.1 ml.
Dist. Water	80.0 ml.	16.0 ml.
10 X PBS	8.8 ml.	1.8 ml.
5% Na HCO_3	0.5 ml.	0.1 ml.
	99.8 ml.	20 ml.

D. Agar Overlay

Dist. Water	25.0 ml.	heat to 56° before adding agar
2 X MEM + B.A.	50.0 ml.	
1% DEAE dextran	1.0 ml.	
Add trypsin (dil.1:20)	2.0 ml.	add immediately before agar
2% agar	25.0 ml.	decrease temp. to 56° after melting - add last.
	103.0 ml	

Δ - heat at 56° for 1/2 hr.

100 X P & S = 1000 units penicillin + 1000 units streptomycin.

Dil. 1→5 pennicillin - Streptomycin mixture

5000 U pen / 5000 U. strep #17-603H
Whitaker M-A Bioprod.

Stock Solutions

10 X PBS (-)

NaCl	8.0 gm	autoclave
KCl	0.2 gm	
Na_2HPO_4	1.15gm	
KH_2PO_4	0.2 gm	
DDW to	100.0 ml	

100 X Ca, Mg Stock

$CaCl_2$	1.0 gm	autoclave
$MgCl_2$	1.0 gm	
DDW to	100.0 ml	

Working Solutions

1 X PBS (-)

pH 7.3

DDW	90 ml	Used before trypsinization; diluent of EDTA, trypsin.
10 X PBS (-)	10 ml	

1 X PBS & B.A.

pH 7.3

DDW	98.3 ml	Used as diluent for making virus dilutions and washing of cells before and after inoculation.
0.5% Phenol Red	0.1 ml	
10 X PBS (-)	10.0 ml	
100 X Ca, Mg	1.0 ml	
35% Bovine Albumin	0.57 ml	

E. Maintenance Medium (liquid overlay)

Distilled Water	48.4
5% Na HCO_3	1.6
2 X MEM + BA	50.0
3 ml/dish	100.0

F. Trypsin/EDTA (Used for subculturing)

1% Trypsin (DIFCO) in PBS	10.0 ml.	(Sterile filter trypsin
2% EDTA in DW	0.5 ml.	with Nalgene unit
DW	80.0 ml.	
10 X PBS	8.8 ml.	
5% Na HCO_3	0.5 ml.	
	99.8 ml.	

Prepare 20 ml at a time.

G. Trypsin for overlay (2 µg/ml)

VMF (Worthington Biochemical)

50 mg/vial
add 25 ml. DW/vial = 2 mg/ml
dispense in 0.5 ml aliquots
freeze at -20° C

before adding to overlay medium - dilute

1:20 in 2 X MEM + B.A. (0.1 ml trypsin + 1.9 ml 2 X MEM)

H. 2% agar - OXOID (Code L28)

2g agar /100 ml DW
dissolve by heating
dispense
sterilize (autoclave)

I. 5% $NaHCO_3$

5g $NaHCO_3$ in 100 ml DW.
autoclave

J. 2% EDTA

2g EDTA in 100 ml D.W.
autoclave

K. 1% DEAE Dextran

1g DEAE dextran in 100 ml D.W.
autoclave

Reagents and Supplies:

10XMEM - GIBCO - #330-1430; with Earle's salts, without L-glutamine and sodium bicarbonate.

DEAE dextran - Pharmacia - 01-900-1-1889-02

Purified Agar - Oxoid Canada, Inc. - L28

L-glutamine - Whitaker M.A. Bioproducts #17-6050 or 17-605C

Hepes (1.0 M) - Whitaker M.A. Bioproducts #17-737A

fetal calf serum - GIBCO #200-6140-mycoplasma tested and virus screened

bovine albumin (35%)
Miles #81-006

penicillin-streptomycin mixture - Whitaker M.A. Bioproduces #17-603H - 5000 U. pen + 5000 U. strep in 20 ml

trypsin for subculturing - DIFCO certified #0152-13, sterile filter before use

trypsin for overlay - Worthington Biochemical - VMF

Nalgene sterilization filter unit (Type S) - #120-0020 (0.20 µM or 0.45 µM)

CENTER FOR DISEASE CONTROL
ATLANTA, GEORGIA
(TELEPHONE 404 329-3591)

ATCH 2425 PROJECT CODE: AA NO. SPEC: 04 NO. TESTS 0060 SUBMITTOR 100

ENDER: DR. DORIS BUCHER, MICROBIOLOGY 16-10
MOUNT SINAI SCHOOL OF MEDICINE
100 ST AND 5TH AVE
NEW YORK, NEW YORK 10029

DC #82-B5010051 DATE RECD. 01 04 85 CONDITION: D PREVIOUS BATCH NONE

EPORTED BY RAT DATE RPTD: 01 11 85 TYPE REPORT: 12

PECIMEN ID	TYPE	SUBMITTORS CODE AND COMMENTS (EG. PATIENT, LAB ID)	RESULTS EGG	PRMK	VIRUS
1	CX	15-2, COLL. 12/10/84, H3N2 STRAIN DESIGNATION: A/NEW YORK/13/84	V		PHL
2	CX	18, COLL. 12/18/84, H3N2 STRAIN DESIGNATION: A/NEW YORK/14/84	V		PHL
3	CX	19, COLL. 12/18/84, H3N2 STRAIN DESIGNATION: A/NEW YORK/15/84	V		PHL
4	E2	17, COLL. 12/18/84, H3N2 STRAIN DESIGNATION: A/NEW YORK/16/84	V		PHL

MMENTS:
ECIMEN RESULT CODES:
= VIRUS ISOLATED PHL = A/PHILIPPINES/2/82-LIKE (H3N2)
= NO VIRUS ISOLATED
ENTRY = TEST NOT DONE

Poliomyelitis – Continued

do not permit this interval, then a single dose of OPV is recommended. For adults incompletely immunized with OPV or IPV, the remaining doses should be given to complete the primary series, regardless of the interval since the last dose or the type of vaccine previously received; either OPV or IPV can be used to complete the series. A single additional dose of either OPV or IPV should be given to travelers who have previously completed a primary series of OPV or IPV. ACIP recommendations on poliomyelitis prevention should be consulted for further details (2).

Reference

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Update: Influenza Activity — United States

Influenza virus type A(H3N2) has been isolated from persons with influenza-like illnesses in outbreaks in Sacramento, California, New York City, and Illinois.

In Sacramento, a kindergarten teacher developed influenza-like illness on December 2, 1984, and similar illness in her husband and son developed on December 5. Also, beginning

(Continued on page 11)

TABLE I. Summary—cases of specified notifiable diseases, United States

Disease	First Week Ending			Cumulative, First Week Ending		
	Jan 5, 1985	Jan 7, 1984	Median 1980-1984	Jan 5, 1985	Jan 7, 1984	Median 1980-1984
Acquired Immunodeficiency Syndrome (AIDS)*	88	97	N	88	97	N
Aseptic meningitis	32	74	76	32	74	76
Encephalitis: Primary (arthropod-borne & unspec.)	6	9	11	6	9	11
Post-infectious	1	4	1	1	4	1
Gonorrhea: Civilian	9,703	13,471	18,213	9,703	13,471	18,213
Military	178	240	369	178	240	369
Hepatitis: Type A	225	276	315	225	276	315
Type B	204	207	207	204	207	207
Non A, Non B	37	46	N	37	46	N
Unspecified	65	62	93	65	62	93
Legionellosis	3	6	N	3	6	N
Leprosy	4	6	2	4	6	2
Malaria	1	15	12	1	15	12
Measles: Total**	3	10	10	3	10	10
Indigenous	3	8	N	3	8	N
Imported	-	2	N	-	2	N
Meningococcal infections: Total	22	41	41	22	41	41
Civilian	22	41	41	22	41	41
Military	-	-	-	-	-	-
Mumps	28	46	59	28	46	59
Pertussis	7	34	12	7	34	12
Rubella (German measles)	6	6	10	6	6	10
Syphilis (Primary & Secondary): Civilian	252	364	510	252	364	510
Military	3	2	6	3	2	6
Toxic Shock syndrome	2	8	N	2	8	N
Tuberculosis	142	213	216	142	213	216
Tularemia	-	2	1	-	2	1
Typhoid fever	3	3	4	3	3	4
Typhus fever, tick-borne (RMSF)	1	-	1	1	-	1
Rabies, animal	30	40	72	30	40	72

TABLE II. Notifiable diseases of low frequency, United States

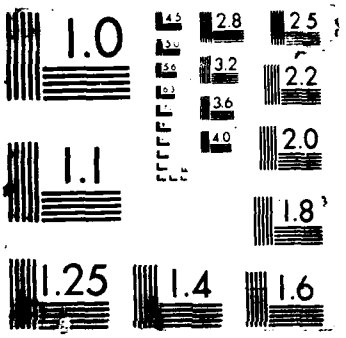
	Cum 1985		Cum 1985
Anthrax	-	Plague	-
Botulism: Foodborne	-	Poliomyelitis: Total	-
Infant	-	Paralytic	-
Other	-	Psittacosis	-
Brucellosis (Mo 1)	1	Rabies, human	-
Cholera	-	Tetanus (Ala 1)	1
Congenital rubella syndrome	-	Trichinosis	-
Diphtheria	-	Typhus fever, flea-borne (endemic, murine)	-
Leptospirosis (N C 2)	2		

*The 1983 reports which appear in this table were collected before AIDS became a notifiable condition.

**There were no cases of internationally imported measles reported for this week.

AD-A195 963 RAPID DETECTION OF ENVELOPED VIRUSES(U) MOUNT SINAI 2/2
MEDICAL CENTER OF THE CITY OF NEW YORK NEW YORK
D J BUCHER 12 MAR 88 DAMD17-85-C-5019

UNCLASSIFIED F/G 6/2 NL


1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2
2.5
2.8
3.2
3.6
4.0

Influenza – Continued

on December 5 and continuing to December 10, an outbreak of influenza-like illness among her students increased the class absentee rate to 50%. Throat swabs from the teacher and her husband and son were collected on December 7. The class was not tested. An influenza type A(H3N2) virus was isolated from the teacher's son.

In New York City, six type A(H3N2) influenza viruses were isolated from inmates at Rikers Island Prison in December. Five isolates were recovered from young adults in the adolescent detention unit of Rikers Island Prison who had influenza-like illnesses from December 17 to December 19. During the outbreak, the number of patients on sick call with upper respiratory illnesses increased from approximately five to 20 daily. In addition, seven type A(H3N2) influenza virus isolates were identified from elderly patients at an extended-care facility in New York City who had influenza-like illnesses in an outbreak that began in mid-December. From December 20 to December 28, there were 30 patients with influenza-like illnesses among the approximately 500 nursing-home residents.

In Illinois, two type A(H3N2) isolates were collected from students in school outbreaks that took place in mid-December in two counties outside Springfield. Four type A(H3N2) isolates were identified from students at the Chicago campus of Loyola University who reported to the student health center in December. Approximately 170 patients and staff members at the Hines Veterans Administration Hospital in Chicago had influenza-like illness in an outbreak that began in mid-December. Type A(H3N2) viruses were identified from each of the five specimens tested in the outbreak.

Isolates of influenza virus type A(H3N2) from sporadic cases have also recently been reported from Arizona, Colorado, Florida, New Jersey, and New Mexico. Type A(H3N2) isolates were reported from Utah in conjunction with school outbreaks in December. Twelve states have now reported isolates of type A(H3N2) influenza virus this season (Arizona, California, Colorado, Florida, Illinois, Nevada, New Jersey, New Mexico, New York, Texas, Utah, and Wisconsin). One type A(H1N1) isolate from Houston, Texas, and type B influenza virus isolates from Hawaii, Illinois, Ohio, and Texas have also been identified.

Reported by P Hom, MD, Sacramento County Health Dept, California Dept of Health Svcs; V Tucci, C Singer, MD, Long Island Jewish Hospital, Nassau County Health Dept, Long Island, D Bucher, PhD, Mt Sinai School of Medicine, H Neu, MD, Columbia Presbyterian Hospital, J Hayes, F Silverstone, MD, New York City, I Spigland, MD, R Cohen, MD, Montefiore Medical Center, New York City, S Beatrice, PhD, S Friedman, MD, New York City Dept of Health; B Haslam, Utah Dept of Health, M Beem, MD, Univ of Chicago Medical School, C Pachuki, MD, Hines Veterans Administration Hospital, M Wu, MD, Loyola University, Chicago, R Murphy, PhD, Illinois Dept of Public Health; C Linnemann, MD, Univ of Cincinnati Hospital, Ohio; P Gross, MD, Hackensack Medical Center, New Jersey; G Meiklejohn, MD, University of Colorado, Denver; E Buff, MS, S Lieb, MS, F Wellings, PhD, Florida Dept of Health and Rehabilitative Svcs; State Epidemiologists and Laboratory Directors; Influenza Br, Div of Viral Diseases, Center for Infectious Diseases, CDC.

Phytophotodermatitis among Grocery Workers — Ohio

On July 5, 1984, a 33-year-old woman presented to an Ohio medical clinic with a bullous, erythematous, nonpruritic, discrete rash of the left forearm of 6 days' duration. An occupational history indicated that she was a cashier at a supermarket. Several co-workers were reported to have had similar rashes that were attributed to handling celery.

The physician alerted the National Institute for Occupational Safety and Health (NIOSH), and a NIOSH medical officer visited the market. A cross-sectional study of all employees was undertaken. Fifty-two (95%) of 55 current full- and part-time employees were interviewed and examined between July and September. Fourteen (27%) of these workers had papular, well-circumscribed rashes confined to the upper extremities, with residual blistering or hyperpigmentation. Dates of rash onset ranged from April through August, with a peak in July. All

HYBRIDOMA PROTOCOL

Immunization - Use Balb/c mice (females).

Inject I.P. 25µg M-protein I.P. in Freund's complete adjuvant. Boost 3 days prior to fusion with 5µg M-protein intravenously through tail (use restrainer). Fusions were made previously with spleens from mice receiving smaller amounts of M-protein; lower numbers of hybridomas were obtained.

SP2/0 cells - (maintain at ∿ 500,000/ml).

Grow cells prior to fusion in D.M.E.M.-HYTH-FBS medium containing 8 - azaguanine. Maintain cells in log phase prior to use.

Fusion - hybridoma

1. Cervically dislocate mouse to sacrifice. Pin mouse to styrofoam board and remove spleen using sterile technique. Place spleen in petri dish containing 5ml Dulbecco's MEM (DMEM). Transfer to second dish containing 5ml DMEM.
2. Shred spleen by tearing apart with two forceps or syringes. (Alternatively, spleen can be shredded by scraping spleen over fine screen). Once separated, use 10cc syringe and pull up cell suspension, breaking up large aggregates and place in 15ml conical test tube. Add additional 5ml DMEM to petri dish to wash out remaining cells and add to tube. USE STERILE TECHNIQUE.
3. Centrifuge cells at 1000 rpm for 10 min. at room temp. Aspirate supernatant.
4. To lyse contaminating red blood cells, add 4.5 ml sterile distilled water to pellet (from #3). Quickly add 0.5ml sterile 10XPBS, pipet up and down several times; ghosts of red blood cells will stick to glass stirring rod or glass pipet. Remove ghosts with stirring rod. Spleen cells are now in 1XPBS. Pellet at 1000 rpm for 10 min. Resuspend in DMEM. Place on ice

5. Wash SP2/0 cells in DMEM three times by centrifuging for 10 min. at 3000 rpm to remove all traces of 8 azaguanine.
[Place polyethylene glycol in 42°C. water bath].

6. While washing SP2/0 cells, count spleen cells (only viable cells). Dilute aliquot of cells 1:10 for counting. The count should be about $1X10^8$ cells/spleen.

7. Count SP2/0 cells (do not dilute).

8. Combine SP2/0 and spleen cells in a 50 ml conical test tube. Add spleen cells and SP2/0 cells at a ratio of 1:10 (SP2/0:Spleen). Utilize all spleen cells.

9. Centrifuge at 1000 rpm for 10 min.

10. Prepare a 30% PEG solution in DMEM from concentrated PEG solution at 42°. (Also incubate aliquot of DMEM-HYTH-FBS at 42°).

11. Aspirate supernatant from SP2/0-spleen cells. Tap tube to loosen pellet, create heavy slurry.

12. Add 0.2 ml 30% PEG solution/10^8 spleen cells. Mix gently by tapping tube. Centrifuge at 900 rpm for 6 min. Allow to sit for 2 min. at room temp. Gently tap tube before next step.

13. Add 5 ml of DMEM-HYTH-FBS gently and centrifuge at 1000 rpm for 5 min Resuspend the cells in 5ml DMEM-HYTH-FBS for every 10^8 spleen cells.

14. Resuspend spleen cells to 30ml for every 10^8 spleen cells. Add 100µl/well to 96 well plate.

15. Incubate at 37° for 24 hr.

16. Add 100 µl HAT medium/well.

17. One week later, aspirate most of supernatant from wells and add DMEM-HYTH-FBS medium.

18. Incubate cells at 37°. Transfer to multiwell plates (24/plate) as clones develop.

[Keep cells on ice during manipulations except where noted].

Subcloning

Hybridoma cells can be subcloned from multiwell dishes or flasks when good cell growth is achieved.

1. Remove thymus(es) from mice or rat. Shred thymus as for spleen in DMEM-HYTH media. Wash 3 times in DMEM-HYTH - FBS centrifuging at 1000 rpm for 10 min each time. Count cells and dilute in DMEM-HYTH-FBS media. Add ~200,000 — 500,000 thymocytes in 100 µl/well of 96 well dish.

2. Dilute hybridoma cells in DMEM except for final dilutions in DMEM-HYTH-FBS; perform two dilutions to provide 10 cells/ml or 20 cells/ml. Dispense 100 µl to each well with stepper syringe. Use separate plate for each dilution and separate plates for each hybridoma.

3. Clones should be visible 5 days following subcloning. When clones cover approx. one-quarter of well with media turning acidic, transfer to multi-well dishes and add 0.5 ml media/well. Screen for reactive clones via ELISA.

Preparation of ascites fluids

Propagate subclones in flasks in DMEM-HYTH-FBS. Pristane-prime mice (retired breeders) 5-7d. I.P. before injecting cells. Have cells growing in log phase for injection. Count cells, centrifuge 10' @ 1000 rpm; resuspend in DMEM-HYTH to a final concentration of $2X10^6$/ml. Inject 0.5 ml I.P. per mouse. Harvest ascites cells sterilely. Add 0.5 ml 2.5% Na citrate/5 ml ascites fluid to prevent aggregation (if cells are not intended for storage in liquid N_2).

Freezing cells

Count cells and centrifuge at 1000 rpm for 10 min. Resuspend in DMEM-HYTH-FBS containing 10% DMSO to a final concentration of $3X10^6$ cells/ml. Place on ice. Dispense in 1.8 ml aliquots. Wrap tubes in cotton and place in Revco to permit slow freezing.

Thawing cells

Quick-thaw tubes at 37°. Centrifuge at 1000 rpm for 10 min and remove supernatant by aspiration. Wash three additional times with DMEM-HYTH-FBS medium. Following final wash, resuspend cells in high fetal bovine serum - DMEM-HYTH-FBS (add additional 10 ml fetal bovine serum to 90 ml DMEM-HYTH-FBS).

Media, Reagents

OPI - 100X

cis-Oxaloacetic acid	1500 mg
pyruvic acid	500 mg
insulin	2000 units

Distilled water to 100 ml.

Sterile filter, aliquot and store at -20°C.

HYTH - hypoxanthine - thymidine

100X working concentration = hypoxanthine 10^{-4}M

thymidine $1.6X10^{-5}$M

- Add 408 mg hypoxanthine (Sigma) to 100 ml distilled water. Stir. Add 1N NaOH to dissolve, if necessary.
- Dissolve 114 mg thymidine in 100 ml distilled water.
- Combine hypoxanthine and thymidine solutions and increase volume to 300 ml with dist. H_2O.
- Adjust pH to 10 with 1N HCl.

Sterile filter, aliquot and store at -20°C.

8-azaguanine - 100 X stock - 10^{-2} M; working con. = 10^{-4} M.

Add 76 mg 8-azoquanine to 35 ml dist. water.

Heat at 37° to dissolve, if necessary.

Adjust pH to 10 with 1N acetic acid.

Increase to final volume of 50 ml.

Sterile filter, aliquot and store at - 20°C

polyethylene glycol solution (∿ 3500 in PBS)

Aminopterin 50X Stock - 4×10^{-5} M; working conc. = 8×10^{-7} M.

Add 17.6 mg aminopterin to one l. dist. water.

Add 1N Na OH until dissolved.

Adjust to pH 7.5-7.8 with 0.5N acetic acid.

Sterile filter, aliquot and store at -20°C.

Dulbecco MEM - HYTH - FBS

Fetal bovine serum (heat inactiv: 56° - 30')	100 ml
penicillin and streptomycin	5.5ml
L-glutamine (MA Biologics, 200 MM)	6 ml
O.P.I. (accord. to ingredient list)	6 ml
HYTH (accord. to ingredient list)	6 ml
NCTC - 109 (GIBCO)	50 ml
Del. MEM (GIBCO #380-2430) (contains hepes)	500 ml

HAT medium (same as above with aminopterin added).

Add one ml aminopterin/50 ml Dulbecco M.E.M.-HYTH-FBS

ELISA Analysis of Influenza Containing Specimens

Optimized Protocol (11/85)

1. Coat plates with 200 µL carbonate buffer containing purified IgG (2 to 5 µg/100 µL).
2. Wash three times with 200 µL PBS-Tween.
3. Postcoat with 200 µL of PBS-Tw containing 0.5% BSA. Incubate for 1 hour at room temperature.
4. Fill row 2 with 100 µL of PBS-Tw with additional 0.18% Triton X100. Fill remaining wells with 100 µL of PBS-Tw with additional 0.95% Triton X100.
5. Freeze-thaw specimen three times and warm in 37°C water bath.
6. Add 100 µL of specimen to row 2 and dilute in twofold increments through row 12.
7. Incubate at 37°C for 1/2 hour.
8. Wash three times with 200 µL PBS-Tween.
9. Add 100 µL rabbit anti-M IgG at a concentration of 40 µg/mL in PBS-Tw and 0.5% BSA to all wells. The IgG was absorbed prior to use (see footnote [a]).
10. Incubate for 1 hour at room temperature.
11. Wash three times with 200 µL PBS-Tween.
12. Add 100 µL of goat anti-rabbit IgG conjugated with alkaline phosphatase at a dilution of 1:750 in PBS-Tween and 0.5% BSA. The conjugate was adsorbed prior to use (see footnote [b]).
13. Incubate for 1 hour at room temperature.
14. Wash three times with 200 µL PBS-Tween.

15. Add 100 µL p-nitrophenol phosphate substrate in diethanolamine buffer.

16. Incubate for 1 hour at room temperature in dark.

17. Stop reaction with 100 µL 1N NaOH and read absorbance with Titertek.

a. IgG is diluted to one mL with PBS-Tw and 0.5% BSA. One mL 'control' gargle specimen is added and held at 5-10°C for 15 min. IgG is then diluted to final concentration of 40 µg/mL; 3% allantoic fluid added, and held an additional 15' at 5-10°C. IgG preparation is clarified by centrifugation at 1,500 rpm for 30 min.

b. Conjugate is diluted to 0.5 mL with PBS-Tw and 0.5% BSA and 0.5mL 'control' gargle added. Conjugate is held at 5-10°C for 15 min and clarified by centrifugation at 1,500 rpm for 20 min. The conjugate is then diluted to the final concentration.

Alkaline Phosphatase Conjugation of Antibodies

Modified from A. Voller, D.E. Bidwell and A. Bartlett (1973) Bull. World Health Organ. 53:55-65 and Kearney et al. (1979). J. Immunol. 123:1548

1. Alkaline phosphatase, Sigma P 5521, 5000 U/5mg protein in crystalline form is centrifuged at 3000 rpm for 10 minutes and the supernatant discarded.
2. One ml purified antibody (2 mg/ml) is added to pelleted alkaline phosphatase.
3. The antibody - alkaline phosphatase mixture is dialyzed overnight at 4°C versus 4 liters PBS, pH 7.4
4. The antibody - alkaline phosphatase mixture is removed from dialysis and 20 µl of 10 glutaraldehyde (EM grade) added. The reaction is allowed to continue for two hours at room temperature with gentle stirring.
5. The preparation is dialyzed versus 4 l. PBS, pH 7.4, overnight at 4°C.
6. The preparation is dialyzed a second time versus 4 l. 0.5 M Tris-HCl, pH 8.0.
7. The sample is brought up to 4.0 ml with 0.5 M Tris-HCl containing 0.001 M Mg, Cl_2, pH 8.0, bovine serum albumin (RIA grade, Sigma A-7888) added to 1.0%, and sodium azide added to 0.04%.
8. The alkaline phosphatase conjugated antibody preparation is stored at 4°C.

Western Blot Protocol

As adapted by S. Popple from Promega Biotec System.

SDS- Polyacrylamide gel electrophoresis -

Polyacrylamide gel electrophoresis of proteins of interest is performed in slab gel electrophoresis system. Protein concentrations applied are in the range of 500 pg to 2 µg.

Electrophoretic transfer -

Electrophoretic transfer of proteins from gel to blotting membranes is performed in a Biorad electroblotting apparatus at 100 watts/0.36 amps for 4 hrs in Transfer Buffer with cooling coil. Promega Biotec membranes or Schleicher and Shull nitrocellulose (BA83, 0.20 µm pore size) work equally well.

Transfer buffer

Trizma (Sigma)	7.5g
Glycine (Sigma)	36.0g
Methanol	500 ml
Distilled water	2000 ml

After electroblotting, nitrocellulose strips can be stored in the Revco in sealed polyethylene bags. Strips must be soaked in TBST buffer (see below) prior to use.

Western Blot

1. Block strips with 15 ml TBST buffer containing 3% BSA and 2% normal sheep serm for two hours, with gentle shaking. Goat serum can be substituted. Match serum to source of secondary conjugated antibody.
2. Remove blocking solution and add 15 ml test antibody diluted in TBST

containing 1% BSA and 1% normal sheep serum. Incubate for 30 minutes with gentle shaking.

3. Remove test antibody and wash with TBST with vigorous shaking for three sequential washes of five min., 30 min., and 10 min.
4. Add alkaline phosphatase conjugated sheep anti-mouse antibodies diluted in 15 ml TBST containing 1% BSA and 1% normal sheep serum. Incubate for 30 min. with gentle shaking.
5. Remove test antibody and wash with TBST with vigorous shaking for three sequential washes of five min., 30 min., and 10 min.
6. Air dry blotted membrane strips on filter paper.
7. Place strips in 5 ml substrate solution in AP buffer (made according to Promega Biotec specifications). The same substrate preparation can be used for visualization of several strips.
8. Stop reaction by immediately placing in stop solution and place in cold overnight.
9. Air dry filters, mount and save.

Antibody dilutions: monoclonal ascites - 1:5000

polyclonal antisera - 1:2000

alkaline phosphatase conjugate (Sigma) - 1:5000

Solutions

TBST Buffer: 10 mM Tris-HCl, pH8.0

150 mM NaCl

0.05% Tween 20

prepare from stock solutions:

10 ml 1 M Tris-HCl, pH8.0

30 ml 5M NaCl

0.5 ml Tween 20

959.5 ml distilled water
1000.0 ml

AP Buffer: 100 mM Tris-HCl, pH9.5

100 mM NaCl

5 mM $MgCl_2$

prepare from stock solutions:

100 ml 1 M Tris-HCl, pH9.5

20 ml 5 M NaCl

10 ml 0.5 M $MgCl_2$

870 ml distilled water
1000 ml

for each 3 nitrocellulose strips: 5 ml AP buffer

33 µl NBT

16.5 µl BClP

Stop Solution: 20 mM Tris-HCl, pH8.0

5 mM EDTA

Prepare from:

10 ml 1 M Tris-HCl, pH8.0

25 ml 0.1 M EDTA

465 ml distilled water
500 ml

11/85

(developed by M. Baer)

Immunofluorescence Analysis (Microscopic) Protocol

Preparation of coverslips containing cell monolayers

1. Autoclave coverslips and place on large petri dishes (100 mm diameter).
2. Add 10 mL growth media containing $1.5X10^6$ CV-1 cells or $2.5X10^6$ MDCK cells.
3. Maintain at 37°C for approximately 4 days to reach confluence.

Infection of cell monolayer

1. Remove media.
2. Wash cells with 5-10 mL PBS.
3. Dilute virus in PBS+Bovine albumin to appropriate level to provide 0.1 to 1.0 PFU/cell in 2.0 mL.
4. Incubate for 30 min at 37°C.
5. Remove virus-containing solution.
6. Add 10 mL maintenance media to each plate.

Preparation of coverslips for analysis

1. Remove media.
2. Carefully remove coverslips with spatula.
3. Acetone fix at -20°C for 10 min.
4. Store at 4°C.

Preparation of cells for use as adsorbent

1. Scrape cells from monolayers (CV-1 or MDCK) of several plates, elute with PBS and centrifuge at 1500 rpm for 10 min.
2. Pour off supernatant and add 2 mL acetone.
3. Vortex several times over 10 min.
4. Centrifuge cells and remove acetone.
5. Resuspend cells in 2 mL PBS for use as adsorbent.

Preparation of antibody and conjugate solutions

1. Dilute antibody and second antibody fluorescein conjugate to appropriate dilutions (1:25-1:50) in PBS containing 8% fetal calf serum and 2% normal goat serum (NGS).
2. Adsorb with acetone treated CV-1 or MDCK cells by addition of 0.2 mL cells per mL diluted antibody or conjugate solution.
3. Vortex every 5 min over 30 min period.
4. Centrifuge solution at 1,500 rpm for 10 min.

Reaction of infected cells with conjugate

1. Add 0.2 mL 10% NGS in PBS per coverslip and incubate 10 min at room temp.
2. Remove NGS by pouring off coverslip and immediately add 0.2 mL antibody solution.
3. Place slides in moisture chamber and incubate for 30 min at 37°C.
4. Clamp coverslips to slides and wash vigorously in PBS for 5 min.
5. Repeat PBS wash in second chamber for additional 5 min.
6. Wash slides in distilled water for 1 min.
7. Air dry coverslips and remove clamps.
8. Add 0.2 mL conjugate solution per coverslip.
9. Place in moisture chamber and incubate for 20 min at 37°C.
10. Attach coverslips to slides via clamps and wash vigorously in PBS for 5 min.
11. Repeat PBS wash in second chamber for additional 5 min.
12. Wash slides in distilled water for 1 min.
13. Air dry coverslips and remove clamps.
14. Place glycerol-buffer* on slide and invert coverslip on glycerol.
15. Store at -20°C in dark a minimum of 18 hr before examining with fluorescence microscope.
16. Photography is performed with Ektachrome 200 color slide film.

*glycerol-buffer: 45 mL glycerol is mixed with 5 mL buffer (buffer consists of 94.5 mL 0.1M Na_2HPO_4 and 5.5 mL 0.1M NaH_2PO_4.)

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Synthetic peptide useful for rapid diagnosis of influenza virus infection. A. Judd, S. Popple, and D. Bucher, U.S. patent application No. 050,633 filed May 14, 1987 by SRI, International, 333 Ravenswood Avenue, Menlo Park, CA 94025

Publication:

D.J. Bucher, I.G. Kharitonenkov, M.W. Khan, A. Palo, D. Holloway and A. Mikhail (1987). Detection of influenza viruses through selective adsorption and detection of the M-protein antigen. J. Immunol. Methods 96:77-85.

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